

**Investigating Calcium binding protein 7
(CaBP7), phosphoinositide signalling and
lysosomes during mammalian cell mitosis**

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Abstract

Calcium binding proteins (CaBPs) are a subfamily of the calmodulin related superfamily of EF hand containing proteins. CaBPs can be further divided into two subgroups (CaBPs 1-5 and CaBP7 and 8) due to differing cation binding properties and because CaBP7 and 8 have a distinct trans-membrane domain at the C-terminal that is essential for determining their subcellular location. CaBP7 and 8 have been shown to interact with Golgi resident Phosphatidylinositol 4-Kinase-III β (PI4KIII β) and to be involved in calcium (Ca^{2+}) regulated Golgi to plasma membrane trafficking pathway. At resting Ca^{2+} levels CaBP7 and 8 interact with PI4KIII β and inhibit its enzymatic function, to prevent phosphatidylinositol 4-phosphate (PI4P) synthesis and vesicular trafficking. At high Ca^{2+} levels another Golgi resident Ca^{2+} -binding protein, neuronal calcium sensor-1 (NCS-1), displaces CaBP7 and 8 from PI4KIII β , stimulating PI4P production and thereby coupling local Ca^{2+} signals to vesicular transport. In addition to this documented trafficking function, a high- throughput RNAi screen identified CaBP7 as an essential factor for successful completion of cytokinesis in HeLa cells. Mitotic cell division is a fundamental biological process required for normal cellular growth, development and aging. Mitotic failure can lead to a state of aneuploidy, which is an accepted driver of cellular transformation and tumorigenesis. Therefore, this thesis specifically focused on CaBP7 with an aim to understand its unique role during mammalian cell mitosis.

When the subcellular localisation of CaBP7 was examined it was found to be present on both the Golgi complex and, unexpectedly, lysosomes. A recent study identified a previously uncharacterised lysosomal pool of PI4KIII β ,

cellular depletion of which disrupted lysosome trafficking and ultimately led to distinctive lysosomal clustering. In an effort to connect these findings, analyses were designed to reveal whether CaBP7 was involved in regulation of lysosomal PI4KIII β . CaBP7 overexpression (inhibition of PI4KIII β) increased clustering of lysosomes in a similar manner to that observed on cellular depletion of PI4KIII β . This result provides evidence to suggest a role for CaBP7 in lysosomal PI4KIII β regulation and lysosome trafficking, which will require further research to fully delineate.

In order to further understand CaBP7 involvement in mitosis, CaBP7 was depleted from cells using shRNAi, which resulted in a 3-fold increase in binucleate cells compared to control cells. Binucleate cells form as a direct consequence of cytokinesis failure implying a functional requirement for CaBP7 during this process. This data replicated findings from the previous large scale RNAi screen and was extended upon significantly in this study through an analysis of a range of PI4KIII β effectors and their influence on mitosis. The same binucleate phenotype was observed with PI4KIII β overexpression suggesting a role for CaBP7 in regulating PI4KIII β during cytokinesis. Localisation studies revealed that CaBP7, PI4KIII β and lysosomes re-distributed together extensively during mitosis implying a link between all three in this process. In particular, at cytokinesis, all three components were localised in discrete clumps flanking either side of the nucleus. Intriguingly this marked re-distribution was lost upon CaBP7 depletion, possibly revealing a mechanistic link to cytokinesis failure. Collectively, data acquired regarding CaBP7, PI4KIII β and lysosomes inferred a role for lysosome positioning during mitosis and to test this

hypothesis experiments were designed to examine a requirement for specific lysosomal activities during cytokinesis. Lysosomes have emerged as Ca^{2+} signalling platforms and this function was assessed using novel genetically encoded Ca^{2+} sensors targeted specifically to these organelles. No Ca^{2+} signals originating from lysosomes during mitotic cell division were detected in these analyses. The other known functions of lysosomes were also examined in these studies. Inhibition of lysosomal catabolism failed to influence mitosis however disruption of lysosomal membrane fusion with the agents GPN and vacuolin-1 induced a significant increase of binucleate cell numbers. Collectively these functional assays suggest a potential requirement for lysosomal membrane fusion during cytokinesis, which would be consistent with a documented function for endosomes during this process. This thesis provides new insights into the role of Ca^{2+} binding proteins, phosphoinositide signalling and, uniquely, lysosomal compartments, during mammalian cell mitosis. It describes an outline for a potentially new regulatory input into mitosis and provides a platform for future detailed examinations of the mechanistic links between CaBP7, lysosomes, lysosomal PI4KIII β activity and PI4P levels during normal cytokinesis in mammalian cells.

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Abbreviations

AF	Autophagic flux
AOBS	Acousto-Optical beam splitter
AP1	Adapter protein complex 1
APC/C	Anaphase- promoting complex/ cyclosome
ANF	Abnormal nuclei frequency
ARF-1	adenosine diphosphate ribosylation factor 1
ALIX	ALG-2 interacting protein X
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BSA	Bovine serum albumin
BP	Base pair
Ca ²⁺	Calcium
CaBP (s)	Calcium binding proteins
CaM	Calmodulin
CDE	Clathrin dependent endocytosis
CDF	Calcium dependent facilitation
CDI	Calcium dependent inactivation
Cdk	Cyclin dependent kinase
CD-M6PR	Cation dependent- Mannose 6 phosphate receptor
cDNA	complementary deoxyribonucleic acid
CDP- DAG	Cytidine diphosphate-diacylglycerol
CICR	Calcium induced Calcium release
CIE	Clathrin independent endocytosis
CI- M6PR	Cation independent- Mannose 6 phosphate receptor
CMA	Chaperone mediated autophagy

CNS	Central nervous system
CPC	Chromosomal passenger complex
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide Triphosphate
E.coli	Escherichia coli
ECL	Enhanced chemiluminescence
Ect2	Epithelial cell-transforming 2
EDTA	Ethylenediaminetetraacetic acid
EE	Early endosome
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complexes required for transport
<i>g</i>	acceleration of gravity
G1	Gap1
G2	Gap2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GEF	Guanosine exchange factor
GFP	Green fluorescent protein
GGA	Golgi- localised, gamma-ear containing ADP-ribosylation factor binding protein
GOLPH3	Golgi- phosphoprotein 3

GPCR	G-protein coupled receptors
GTP	Guanosine triphosphate
GST	Glutathione S- transferease
IAP	Inhibitor of apoptosis
ILV	Intraluminal vesicles
INCEP	Inner centrosome protein
IP ₃	Inositol triphosphate
IP ₃ R	Inositol triphosphate receptor
JACoP	Just another colocalisation plugin
H ⁺	Hydrogen ion
HA	Human influenza hemagglutinin
HCl	Hydrochloric acid
HeLa	Henrietta Lacks
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
KCl	Potassium chloride
kDa	kilo Dalton
LAMP1	Lysosome associated membrane protein -1
LCD	Lysosome cell death
LDL	Low density lipoprotein
LE	Late endosome
LMP	Lysosomal membrane protein
LSD	Lysosome storage disease
M6PR	Mannose 6 phosphate receptor
M	Mitosis
MA	Macroautophagy

mCh	mCherry
MCU	Mitochondrial calcium uniporter
Mg ²⁺	Magnesium
MgCl	Magnesium Chloride
Mklp	Mitotic kinesin like protein
uM	micromolar
MVB	Multivesicular bodies
Na ⁺	Sodium
NaCl	Sodium Chloride
Na ₂ HPO ₄	Sodium phosphate dibasic anhydrous
NaH ₂ PO ₄	Sodium phosphate monobasic monohydrate
NCS-1	Neuronal calcium sensor -1
NCX	Na ⁺ / Ca ²⁺ exchanger
nM	nanomolar
PA	Phosphatidic Acid
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PH	Pleckstrin hology
pH	(log10 of [H ⁺])
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PI4P	Phosphatidylinositol (4)-phosphate
PI (4,5) P ₂	Phosphatidylinositol 4,5-bisphosphate

PI4KIII β	Phosphatidylinositol 4-kinase III beta
PKD	Protein kinase D
Plk	Polo like kinase
PM	Plasma membrane
PMCA	Plasma membrane calcium ATPase (adenosine triphosphatase)
PS	Phosphatidylserine
RFP	Red fluorescent protein
ROC	Receptor operated channel
ROCK	Rho activated kinase
ROI	Region of interest
Rpm	Revolutions per minute
RT-PCR	Reverse transcription- polymerase chain reaction
RyR	Ryanodine receptors
S	Synthesis
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SERCA	sarcoendoplasmic reticulum (SR) calcium transport ATPase (adenosine triphosphatase)
SOC	Super Optimal broth with Catabolite repression
shRNAi	short hairpin ribonucleic acid interference
SMOC	Second-messenger operated channel
SNARE	soluble N-ethylmaleimide sensitive factor attachment protein receptors
SR	Smooth endoplasmic reticulum
TA	Tail anchor

TAE	Tris base, acetic acid and EDTA
TBS	Tris buffered saline
TGN	Trans- Golgi Network
T _m	Melting temperature
TMD	Transmembrane domain
TPC	Two pore channel
TRP	Transient receptor potential
TRPML	Transient receptor potential cation channel, mucolipin subfamily
TSE	Tubular sorting endosome
Tsg	tumour susceptibility gene
UV	Ultraviolet
VAMP7	Vesicle associated membrane protein 7
V-ATPase	Vacuolar ATPase (adenosine triphosphatase)
VGCC	Voltage gated calcium channel
VOC	Voltage operated channel
Vps	vacuolar protein sorting-associated protein
YFP	Yellow fluorescent protein

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Chapter 1

Introduction

1.1. Calcium signalling

The calcium ion (Ca^{2+}) is the most universal intracellular second messenger and is involved in a wide range of physiological functions and cellular processes (Bootman & Roderick, 2011; Clapham, 2007). Its functions extend to almost all aspects of cell physiology and influences processes as diverse as fertilization, proliferation, gene transcription, metabolism, muscle contraction, exocytosis, ion channel modulation and cell death (Berridge, Bootman, & Roderick, 2003; Berridge, Lipp, & Bootman, 2000). It is somewhat surprising that a simple cation with two charges can regulate so many essential cellular functions. Ca^{2+} is the messenger however it is the underlying cellular machinery that detects its presence and modulates its release, which allows such diverse modes of signalling. Mammalian cells express large numbers of Ca^{2+} channels, pumps and exchangers that can generate cytoplasmic Ca^{2+} transients of varying duration (temporal parameter), intensity (amplitude) and location (spatial parameter). Interplay between these proteins makes an almost endless number of distinct Ca^{2+} signals available to the cell. The second component of the signalling machinery consists of large numbers of dedicated Ca^{2+} binding/sensory proteins each of which has distinct biochemical and cellular properties and which is responsible for decoding a unique class of Ca^{2+} signal. The Ca^{2+} sensors therefore couple individual signals to a specific cell signalling pathway and alteration in cellular behaviour. Using this complex 'calcium toolkit' (Berridge et al., 2000; 2003) mammalian cells are therefore able to utilise Ca^{2+} to drive the huge variety of processes described above.

The Ca^{2+} concentration ($[\text{Ca}^{2+}]$) varies immensely between the extracellular space and interior cytosol of a cell. The external Ca^{2+} pool ($\sim 1\text{-}2\text{ mM}$) is some 20,000 folds higher than the resting cytosolic Ca^{2+} concentration ($\sim 100\text{ nM}$) and cells expend large amounts of energy in order to maintain this gradient. This large difference is generated under resting conditions by the concerted action of Ca^{2+} -channels, Ca^{2+} -exchangers and Ca^{2+} -buffers. Additionally Ca^{2+} can be sequestered into the lumen of cellular organelles such as the endoplasmic reticulum (ER) (Berridge, 2002; Bygrave & Benedetti, 1996), mitochondria (CHANCE, 1965; ROSSI & LEHNINGER, 1964) and lysosomes (Christensen, Myers, & Swanson, 2002; Lloyd-Evans et al., 2008). Ca^{2+} sequestering organelles have a substantial Ca^{2+} capacity, which makes them significant Ca^{2+} buffers upon a cytosolic Ca^{2+} rise. These intracellular organelles are also fundamentally important Ca^{2+} signalling platforms and this will be discussed in detail in the proceeding sections.

In the majority of cases Ca^{2+} signalling is initiated upon elevation of the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) from resting ($\sim 100\text{ nM}$) to $1\text{ }\mu\text{M}$ or more and Ca^{2+} transients can originate from either external or internal pools. Ca^{2+} entry from the extracellular medium is driven by the large chemical gradient across the plasma membrane (PM) via different classes of Ca^{2+} -selective channels such as voltage operated channels (VOCs), receptor operated channels (ROCs), second-messenger operated channels (SMOCs) or transient receptor potential ion- channels (TRPs) (Bootman & Roderick, 2011).

The principal internal store for Ca^{2+} release is the ER (sarcoplasmic reticulum (SR) in muscle tissue) via two distinct classes of Ca^{2+} -channel. Inositol 1,4,5-

trisphosphate receptors (IP₃Rs) and ryanodine receptors (RyRs) are large multimeric membrane proteins that are ubiquitously expressed (Berridge, 2002). The activation of these receptors is driven by the production of the intracellular second messengers inositol 1,4,5-trisphosphate (IP₃) and cyclic ADP ribose (cADPR) respectively. Plasma membrane G-protein coupled receptors (GPCRs) are activated by extracellular agonists to modulate cytoplasmic levels of both IP₃ and cADPR by regulating the activity of the enzymes phospholipase C and CD38 respectively. Both receptor types are additionally sensitive to Ca²⁺ itself which underlies a Ca²⁺ induced Ca²⁺ response (CICR) mechanism that can elicit rapid propagation and amplification of an initial signal (Berridge, 2002; Petersen, Tepikin, & Park, 2001). Mitochondria are also considered of importance as Ca²⁺ storage organelles and release of Ca²⁺ in this instance occurs via mitochondrial Na⁺/Ca²⁺ and H⁺/Ca²⁺ exchangers that are located on the outer mitochondrial membrane (Rizzuto, De Stefani, Raffaello, & Mammucari, 2012). Whether [Ca²⁺]_i increase by influx of extracellular Ca²⁺ or release from intracellular stores its presence is detected by specific Ca²⁺ binding/sensing proteins, which bind to Ca²⁺ through unique Ca²⁺ binding motifs such as the EF hand. Ca²⁺ sensing proteins are distinguished from other Ca²⁺ binding proteins such as Ca²⁺ buffers by their relatively high affinities for Ca²⁺, unique subcellular localisations and typically extensive conformational changes that are induced on Ca²⁺ binding and which drive interaction with downstream target proteins (Lewit-Bentley & Réty, 2000).

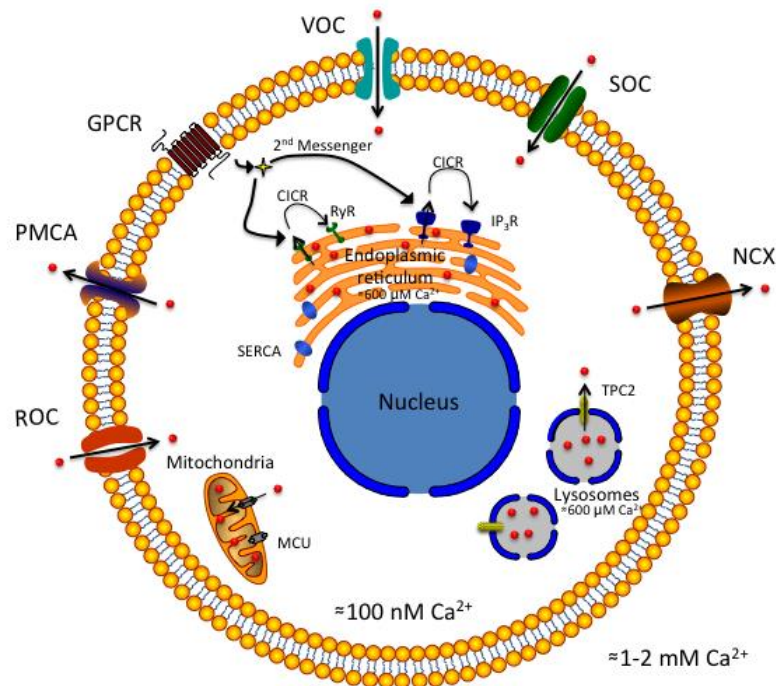


Diagram 1.1. Overview of Ca^{2+} signalling in the cell. A cytosolic Ca^{2+} increase is the typical pathway for triggering a Ca^{2+} signalling cascade. Ca^{2+} from the extracellular medium can enter the cell via PM channels such as voltage-gated channels (VOC), store-operated channel (SOC) or receptor-operated channels (ROC). G-protein coupled receptors (GPCRs) stimulated by extracellular agonists couple to intracellular production of soluble secondary messengers such as IP_3 or cADPR which can diffuse through the cytosol and activate endoplasmic reticulum (ER) resident Ca^{2+} channels: Inositol 1,4,5-trisphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs), respectively. Both receptor classes are additionally regulated by Ca^{2+} itself and this characteristic is important for regenerative propagation of the signal by a mechanism known as Ca^{2+} induced Ca^{2+} release (CICR). Ca^{2+} release from lysosomes is thought to be facilitated by a nicotinamide adenine dinucleotide phosphate (NAADP)-sensitive membrane channel the identity of which could be a family of recently discovered two pore channels (TPCs). Ca^{2+} signals can be terminated via channels on Ca^{2+} storage organelles including the ER resident sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) and mitochondrial Ca^{2+} uniporter (MCU) present on mitochondria. Cytosolic Ca^{2+} is also extruded back into the extracellular medium by the plasma membrane Ca^{2+} -ATPase (PMCA) and by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX).

During the course of a Ca^{2+} transient, buffers and channels are in place to counteract the increased intracellular Ca^{2+} concentration. Prolonged elevations in $[\text{Ca}^{2+}]_i$ will eventually induce activation of cell death programs and therefore every cell treads a fine line when utilizing Ca^{2+} for normal cell signalling activities. Ca^{2+} buffers and extrusion pumps/channels permit the cell to revert back to resting cytosolic Ca^{2+} levels (~ 100 nM) rapidly. The PM Ca^{2+} ATPase (PMCA) and $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX), SR Ca^{2+} ATPase (SERCA) and the mitochondrial Ca^{2+} uniporter (MCU) are the primary mediators of Ca^{2+} removal from the cytoplasm (Berridge et al., 2003).

Ca^{2+} signals begin as a spatially restricted increase in concentration around an open channel protein, often referred to as an elementary Ca^{2+} signal. Most Ca^{2+} signalling events function through such restricted elementary signals, which allow for highly tuned spatio-temporally restricted and specific control. Elementary signals are also the building blocks of larger, more extensive and longer-lived Ca^{2+} oscillations and waves, collectively termed 'global' Ca^{2+} signals. Ca^{2+} signals can also be generated with pulsatile characteristics, that is, they exhibit regenerative properties, creating unique patterns of repetitive Ca^{2+} spikes. The specific spatio-temporal characteristics of a particular type of Ca^{2+} signal are integrated and processed by dedicated Ca^{2+} sensing proteins (Berridge, 1997).

1.1.1. EF hand containing Ca^{2+} binding/ sensing proteins

As elaborated above, Ca^{2+} is a key second messenger in the cell that regulates nearly all aspects of life. This is possible due to the evolution of families of specialised Ca^{2+} binding proteins. Ca^{2+} binding proteins can react

rapidly and specifically to even very fast Ca^{2+} signals through their Ca^{2+} binding properties (Schwaller, 2009). There are currently six characterised unique Ca^{2+} binding protein motifs including the EF hand, C2 domain (Rizo & Südhof, 1998) and the endonexin fold (Kourie & Wood, 2000). The most prevalent Ca^{2+} responsive protein motif is the EF hand (Schwaller, 2009). This motif was first discovered in parvalbumin, a small calmodulin related protein (Kretsinger & Nockolds, 1973). There are at least sixty six subfamilies of EF hand containing proteins including the neuronal calcium sensor (NCS) family, S100 family, calpains, troponin C and calcineurins (Kawasaki, Nakayama, & Kretsinger, 1998). The EF hand module (Diagram 1.2.) consists of a helix-loop-helix structure, whereby a 12 amino acid loop binds to Ca^{2+} through coordination by conserved glutamic acid (Glu) or aspartic acid (Asp) residues (Lewit-Bentley & Réty, 2000). It has been determined that not all EF hands bind Ca^{2+} and various point mutations can lead to inactive EF hands, which are only present for structural maintenance and protein stabilization. For instance, loss of glycine at position 6, deletion of residues, the introduction of positively charged amino acids or the loss of Glu or Asp in the EF hand loop can all result in loss of Ca^{2+} binding capacity (Haeseleer, Imanishi, Sokal, Filipek, & Palczewski, 2002). Additionally some EF hands bind magnesium ions (Mg^{2+}), which can be important for structural integrity and may influence Ca^{2+} binding to other EF hands within the same protein. These dual $\text{Mg}^{2+}/\text{Ca}^{2+}$ binding EF hands are therefore likely to have functionally important roles (Aravind et al., 2008; Gifford, Walsh, & Vogel, 2007).

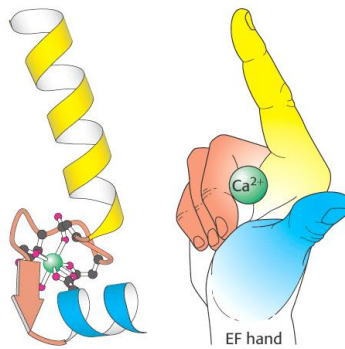


Diagram 1.2. The EF hand – a Ca^{2+} binding domain. A ribbon representation of a Ca^{2+} bound (green sphere) EF hand (left) and its resemblance to a human hand (right) with extended forefinger and thumb (Taken from: Stryer J.M, Berg L. and Tymoczko J.L., 2001. *Biochemistry*. 5th ed. New York: W.H. Freeman).

Active EF hand motifs usually appear in tandem within a protein sequence or EF hand containing proteins dimerise however pseudo/ inactive EF hands also exist. The family of EF hand containing proteins can be grossly divided into two subgroups, the Ca^{2+} sensors and Ca^{2+} buffers (Skelton, Kördel, Akke, Forsén, & Chazin, 1994). Sensors, which are involved in cell signalling events, usually have low Ca^{2+} binding capacity, high binding affinities and undergo a significant conformational shift upon Ca^{2+} binding. These properties distinguish them from Ca^{2+} buffers which have high binding capacities, low affinities and do not typically exhibit extensive conformational alterations (Yap, Ames, Swindells, & Ikura, 1999). Changes in protein conformation expose binding sites on the sensor protein that couple a Ca^{2+} signal to interactions with downstream signalling molecules. Ca^{2+} buffers, although not directly involved in regulation of a particular cell signalling pathway, are nonetheless essential for shaping and localising Ca^{2+} transients and maintaining resting Ca^{2+} levels (Bootman & Roderick, 2011; Tepikin, 2001).

1.1.2. Calmodulin

Calmodulin (CaM) is an ancient protein that is the primordial ancestor and most extensively studied member of the EF hand containing families. It is expressed in all eukaryotic cells and has a broad involvement in various physiological functions including growth, proliferation and movement (Chin & Means, 2000). CaM displays a 100% amino acid identity among vertebrates (Friedberg, 1990). It is a relatively small protein (16.7 kDa) with four active Ca^{2+} -binding EF hands, arranged into two globular lobes (EF hand 1 and 2 forming the N-terminal lobe, EF hand 3 and 4 the C-terminal lobe) connected by a short central flexible linker region (Chin & Means, 2000). This lends CaM a dumbbell like tertiary structure (Diagram 1.3.). In the absence of Ca^{2+} or resting Ca^{2+} apo-CaM adopts a closed conformation with both EF hands of the N-terminal lobe closely packed. Under these conditions the C-terminal lobe displays a semi-open conformation whereby a hydrophobic patch is partially accessible to solvent and mediates interaction with a limited subset of target proteins (Swindells & Ikura, 1996). Upon receipt of a Ca^{2+} signal and elevation of $[\text{Ca}^{2+}]_i$, Ca^{2+} ions bind to the EF hand loops of CaM causing a large structural re-organisation. CaM switches to an open conformation around the flexible linker region exposing hydrophobic surface residues to mediate interaction with a wide range of target proteins (Zhang, Tanaka, & Ikura, 1995). Ca^{2+} dependent activation of CaM was first studied in wound healing processes in fibroblasts (Hahn, DeBiasio, & Taylor, 1992). It has since been studied in more detail and shown to regulate many different cellular proteins and cellular activities both positively and negatively and in Ca^{2+} dependent and independent manners (Chin & Means, 2000).

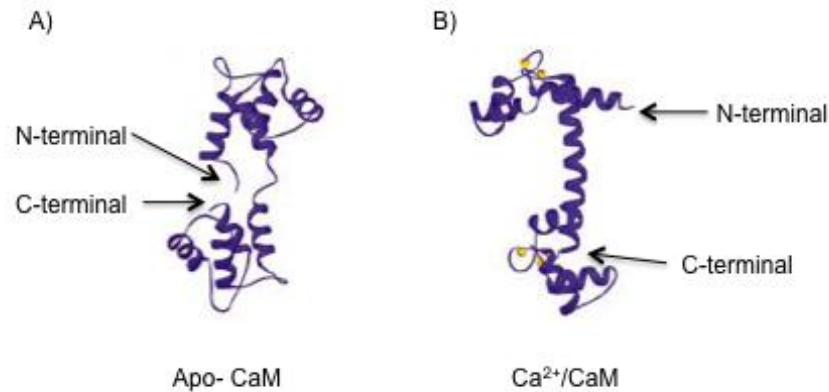


Diagram 1.3. Crystal structures of Ca²⁺ unbound (apo-CaM) and Ca²⁺-bound (Ca²⁺/CaM) CaM.

Ribbon representation of the conformational difference between apo-CaM and Ca²⁺-CaM. A) Apo-CaM: the N-terminal lobe adopts a closed conformation, whereas the C-terminal lobe EF hands are partially- open; B) Ca²⁺/CaM: upon Ca²⁺ binding (yellow spheres) CaM undergoes a dramatic conformational change exposing hydrophobic interaction surfaces. (Taken from: Openwetware.org. 2015. Measuring Calcium in vitro. [ONLINE] Available at: [http://openwetware.org/wiki/20.109\(S07\):_Measuring_calcium_in_vitro](http://openwetware.org/wiki/20.109(S07):_Measuring_calcium_in_vitro) [Accessed 03 August 15]).

Apo-CaM has been characterised to bind fifteen proteins including enzymes, actin-binding proteins, cytoskeletal elements and certain receptors and ions channels (Chin & Means, 2000; Jurado, Chockalingam, & Jarrett, 1999). Binding motifs identified for apo-CaM are the IQ motif (found in myosin, neuromodulin, neurogenin, on VGCCs, etc.) and the catalytic subunit of glycogen phosphorylase b kinase (*PbK*) (Jurado et al., 1999).

Apo-CaM can also function as a VGCC accessory subunit, whereby it is constitutively bound to the cytoplasmic domain of the channel in a Ca²⁺ independent fashion. On Ca²⁺ binding, CaM undergoes a conformational change and this enables interaction with a discrete domain of the channel to

regulate gating resulting in either Ca^{2+} dependent inactivation (CDI) or Ca^{2+} dependent facilitation (CDF). CaM has been shown to elicit CDI and CDF of VGCCs such as $\text{Ca}_v1.2$ - and $\text{Ca}_v2.2$ - type channels (Halling, Aracena-Parks, & Hamilton, 2006; Zühlke, Pitt, Deisseroth, Tsien, & Reuter, 1999). This phenomena has also been observed for the small- conductance Ca^{2+} activated potassium channels (SK channels) (Xia et al., 1998). There are also examples whereby Ca^{2+} - CaM inhibits ion channel opening including all three subtypes of the RyRs (Pucéat, 2010). In addition, the IP_3R and some G-protein coupled receptor kinases are also negatively regulated by Ca^{2+} - CaM (C. W. Taylor & Laude, 2002).

CaM is a ubiquitously expressed protein found at relatively high concentrations within most cell types that exhibits a high degree of sequence conservation across species and which is involved in many fundamental physiological functions. CaM represents the evolutionary template for small EF-hand containing Ca^{2+} sensors and large numbers of related proteins have evolved from this. All of these proteins resemble CaM to some greater or lesser extent but have been shown to have specialised functions distinct from those of CaM. It is interesting to note that the evolution of increasingly complex animals correlates with the appearance of larger and more diverse numbers of CaM related small EF hand containing proteins.

1.1.3. Calcium binding proteins (CaBPs)

CaBPs are a subfamily of six distinct CaM related proteins which first arose in vertebrates (CaBPs 1, 2, 4, 5, 7 and 8, with 9 possible expressed proteins based on alternative splicing of CaBP1 (3 variants) and CaBP2 (2 variants),

(Haeseleer et al., 2000; McCue, Haynes, & Burgoyne, 2010b). This suggests that CaBPs serve functions specific to complex higher organisms. Based on sequence homology and number of EF hands, CaBPs are regarded as CaM related proteins (McCue, Haynes, & Burgoyne, 2010a). As with CaM, CaBPs possess 4 EF hands in a similar N- and C-terminal pairing, however, in contrast to CaM, CaBPs have combinations of inactive and active EF hands (Chin & Means, 2000). The family of CaBPs can be divided into 2 groups based on the pattern of inactive and active EF hand positioning. CaBPs 1-5 possess an inactive EF hand 2 and remaining three active EF hands (1, 3 and 4). In comparison CaBP7 and 8 have active EF hands 1 and 2 and inactive EF hands 3 and 4 (Burgoyne & Haynes, 2010; Haeseleer et al., 2000; Mikhaylova et al., 2009).

The CaBP family have been defined mainly as neuronal specific Ca^{2+} sensors (Haeseleer et al., 2000), and research has focused on their functions in neurons, particularly those of sensory systems. Nonetheless, the human protein atlas project database suggests nearly all CaBPs to be more ubiquitously expressed in both neuronal and non-neuronal tissues (www.proteinatlas.org). CaBP1 and 2 contain a canonical *N*-myristoylation motif with a glycine residue at position 2 of the protein that is myristoylated allowing these proteins to associate with the PM and Golgi apparatus (Haeseleer et al., 2000; Haynes, Tepikin, & Burgoyne, 2004). CaBP7 and 8 can also associate with the PM and Golgi however this is not acylation dependent and is instead facilitated by a novel C-terminal transmembrane domain (McCue, Burgoyne, & Haynes, 2009). The different domain structures of CaBPs and CaM are summarised in Diagram 1.4.

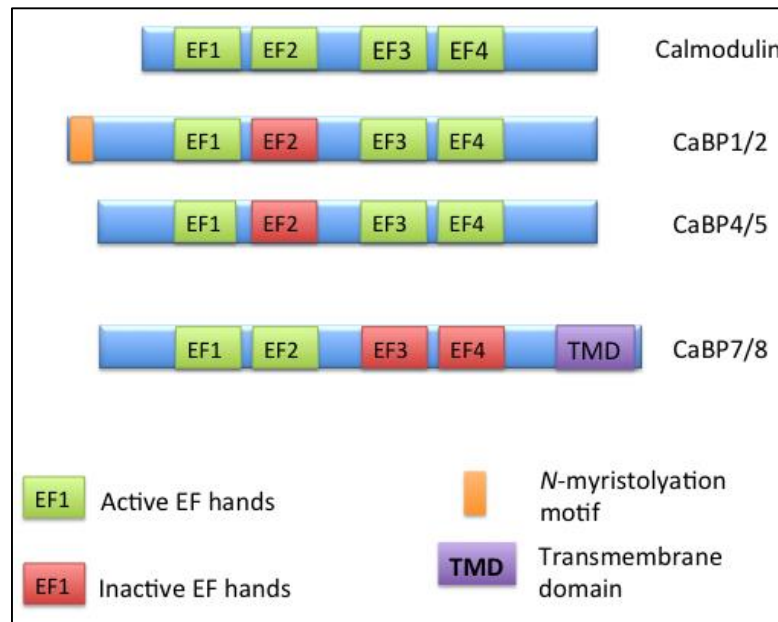


Diagram 1.4. Domain structure of CaBPs compared to their ancestor CaM.

Functional EF hands are indicated in green, whereas red EF hands are non-functional. CaBP1 and 2 possess a *N*-myristoylation motif shown in orange at the N-terminal. CaBP7 and 8 possess a hydrophobic transmembrane domain (TMD) at the C-terminal shown in purple.

1.1.4. CaBPs 1-5

There are three splice isoforms of CaBP1: CaBP1 long, CaBP1 short and Caldendrin. Caldendrin, the longest splice variant contains a unique N-terminal extension but lacks the *N*-myristoylation motif (Haeseleer et al., 2000). Caldendrin and CaBP1 have been studied for their function in modulating neuronal Ca^{2+} channels such as voltage gated $\text{Ca}_v2.1$ P/Q-type (A. Lee et al., 2002) and $\text{Ca}_v1.2$ L-type channels (Oz, Tsemakhovich, Christel, Lee, & Dascal, 2011; Zhou, Yu, McCoy, & Lee, 2005), transient receptor potential channel-5 (TRPC5) (Kinoshita-Kawada et al., 2005) and IP_3Rs (Haynes et al., 2004; Kasri et al., 2004; C. Li et al., 2013).

Interestingly, CaM is also known to interact with the same channels and studies have shown that CaBP1 can compete with CaM for binding to such shared targets. Although CaBPs and CaM share common target interactions the targeting mechanisms, interaction motifs and modes of regulation in these instances have been found to be highly unique. CaBPs therefore are likely to exert essential non-redundant, functions (Oz et al., 2013; Rieke, Lee, & Haeseleer, 2008; P. S. Yang, Johny, & Yue, 2014). For example CaBP1 and CaM have both been demonstrated to interact with and regulate the activity of the Cav1.2 L-type Ca^{2+} channel in *Xenopus* oocytes at partially overlapping sites (Oz et al., 2013). Ca^{2+} - CaM provides a negative feedback loop via Ca^{2+} dependent inactivation (CDI) (Zühlke et al., 1999) whereas CaBP1 strongly suppresses the CDI effect on Cav1.2 by competing with CaM (A. Lee et al., 2002; Oz et al., 2013). Another role of CaBP1 is the regulation of the IP₃R1. CaBP1 interacts with the cytoplasmic N-terminal tail of the IP₃R1 to inhibit IP₃ evoked Ca^{2+} release by reducing channel opening rate (U. Acharya, Mallabiabarrena, Acharya, & Malhotra, 1998; Colanzi, Sütterlin, & Malhotra, 2003; Haynes et al., 2004; C. Li et al., 2013; Shaul & Seger, 2006). CaBP2 has two splice isoforms CaBP2 long (CaBP2L) and CaBP2 short (CaBP2S) which are somewhat poorly characterised (Haeseleer & Palczewski, 2002). CaBP2 is expressed in the retina and hair cells of the inner ear (Haeseleer & Palczewski, 2002) and mutations in CaBP2 that generate a truncated protein have been implicated in moderate/severe hearing impairments in humans due to altered Ca^{2+} binding properties and diminished ability to suppress Cav1.3 Ca^{2+} channel current density (Schrauwen et al., 2012). Furthermore CaBP2S has been shown to activate

CaM Kinase II, an important kinase in mediating signalling cascade for learning and memory (Haeseleer et al., 2000).

CaBP3 has been designated as a pseudo gene, since expression of this protein is undetectable, whereas its mRNA is present in cells (Haeseleer et al., 2000).

Much more research has been conducted on CaBP4, which is a 45 kDa, ubiquitously expressed protein lacking an *N*-myristolation motif. It is expressed in the retina and auditory inner ear cells where it modulates VGCCs (Haeseleer et al., 2000). Biochemical assays confirmed the interaction between CaBP4 and unc119 in photoreceptor terminals (homologue to MRG4- a photoreceptor protein), which suggested it might play a role in neurotransmitter release and photoreceptor synapse maintenance (Haeseleer, 2008). CaBP4 knockout mice exhibit retinal abnormalities resembling, but not phenocopying, incomplete congenital stationary night blindness (CSNB), which is an X-linked non –progressive retinal disorder (Haeseleer et al., 2004). Detailed analyses revealed the importance of CaBP4 in regulating the activity of the Cav1.4 channels in the photoreceptor cells and further investigations have clarified that CaBP4 mutations do not cause CSNB but rather a CaBP4 specific defect that has been termed congenital cone-rod synaptic disorder (Khan, 2014).

Studies on CaBP5 are incomplete and still require much more investigation. CaBP5 is highly expressed in hair cells and the inner ear cells and is also predicted to regulate VGCCs. In CaBP5 knockout mice a reduced light sensitivity of the retinal ganglion cells was observed implying CaBP5 to regulate visual activity (Rieke et al., 2008). Furthermore a Ca²⁺ independent

interaction between CaBP5 and MUNC-18 has been described (Sokal & Haeseleer, 2011). Munc- 18 is a well-established protein involved in the soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) complex formation and in regulating synaptic vesicle fusion to the PM. Since CaBP5 over expression had an effect on neurotransmitter release, it is likely that CaBP5 regulates synaptic fusion events. CaBP5 as CaBP4 lacks membrane targeting motifs or modifications and is cytosolic in localisation (Haeseleer et al., 2000).

1.1.5. CaBP7 and CaBP8

CaBP7 and 8 share limited homology with the other CaBPs. Phylogenetic studies revealing the evolution of CaBPs demonstrated that CaBP7 and 8 form an evolutionary sub-branch distinct from CaBPs 1-5, indicating that these proteins emerged and evolved differently from the other CaBPs (McCue, Haynes, & Burgoyne, 2010b). CaBP7 and 8 possess 4 EF hands, however only two EF hands are active which are located in the N-terminal whereas the C-terminal hands are both inactive. This varies from the pattern of the active and inactive EF hands found in CaBPs 1-5. Additionally CaBP7 and 8 have a unique 38 aa C-terminal hydrophobic extension which resembles the transmembrane domains found in the tail-anchored class of membrane proteins, which is crucial for their correct localisation (Hradsky et al., 2011; McCue et al., 2009; Mikhaylova et al., 2009). Co-localisation experiments with appropriate markers identified CaBP7 and 8 to reside at the Trans-Golgi- network (TGN) and PM (McCue et al., 2009; Mikhaylova et al., 2009). The Ca^{2+} binding capabilities of these proteins have been measured

and CaBP7 and 8 show similar high Ca^{2+} binding affinities of ~230 nM and ~180 nM respectively (Mikhaylova et al., 2009) consistent with a Ca^{2+} sensing role.

Since these are relatively recently discovered proteins there is limited data available regarding their cellular functions. CaBP7 and 8 have been shown to interact with and negatively regulate PI4KIII β at the TGN at resting $[\text{Ca}^{2+}]_i$ (~100 nM). When $[\text{Ca}^{2+}]_i$ is elevated, neuronal calcium sensor -1 (NCS-1) competes for the CaBP7 and 8 interaction site and activates PI4KIII β (Mikhaylova et al., 2009). Upon stimulation, PI4KIII β phosphorylates phosphatidylinositol (PI) to phosphatidylinositol- 4- phosphate (PI4P), which in turn regulates vesicle transport from the TGN (A. Balla & Balla, 2006; Haynes, Thomas, & Burgoyne, 2005; Hilfiker, 2003; Zhao, Várnai, Tuymetova, Balla, & Tóth, 2001). Therefore the interplay between CaBP7 and 8 and NCS-1 represents a possible novel, Ca^{2+} controlled, checkpoint for TGN vesicular transport events. CaBP8 has been demonstrated to regulate N-, L- and P/Q- type VGGCs on the PM of bovine chromaffin cells and therefore even though these CaBP isoforms are more distantly related to the other family members there does appear to be some retention of function. Interestingly, the inhibition of these currents, as for CaBP7 and 8 regulation of PI4KIII β , were Ca^{2+} independent since CaBP8 wild type as well as EF hand mutants all repressed channel activity (Shih, Lin, Cheng, Liao, & Pan, 2009).

In a high-throughput RNAi screen searching for regulators of mammalian cell mitosis, CaBP7 was identified by Neumann *et al.* to play a key role in cytokinesis, which is the final stage of mitotic cell division. In this study

CaBP7 was one of a handful of candidate proteins selected for further detailed analysis including time-resolved 4D-microscopy. These data showed that CaBP7 depleted HeLa cells could complete mitosis normally but that at cytokinesis, even though an intercellular bridge was formed, this structure failed to sever and the nascent daughter cells eventually coalesced back into a single binucleate cell (Neumann et al., 2010). If the same cell was tracked through a second round of mitosis, there was an identical late division failure with the generation of a single tetraploid cell. Cytokinesis failure in this manner is particularly relevant for human health as the state of aneuploidy (abnormal amounts of nuclear material within a single cell) is now an accepted trigger of cellular transformation (Kops, Weaver, & Cleveland, 2005; Pfau & Amon, 2012; Rajagopalan & Lengauer, 2004; Ricke & van Deursen, 2013).

An important role for the small EF hand protein CaM has already been established at several stages of mitosis in model vertebrate systems. The first evidence implicating CaM in cell division was proposed from observations of CaM localisation with the mitotic apparatus and in close proximity to the spindle poles. Subsequent studies confirmed CaM to regulate cell cycle progression (Chafouleas, Bolton, Hidaka, Boyd, & Means, 1982; Choi & Husain, 2006; G. P. Reddy, Reed, Sheehan, & Sacks, 1992). As part of these studies CaM inhibitors were applied and cell cycle arrest, in addition to DNA synthesis and replication inhibition was observed with the accumulation of binucleate cells. Cdk2 activity, which is essential for cell division progression, was also demonstrated to be reduced upon CaM inhibition. CaM has also been shown to directly bind to cell cycle proteins,

namely facilitating cyclin D1 and Cdk4 binding and permitting nuclear translocation of this complex (Choi & Husain, 2006). A recent study also established a CaM specific role during cytokinesis where it co-localises with Aurora B kinase at the midbody and regulates Aurora B protein levels. Aurora B kinase regulates cytokinesis as it stabilises the intercellular bridge to ensure proper chromosome segregation. As part of this study the effect of CaM inhibition on human adenocarcinoma cell growth was examined, and interestingly CaM inhibition led to tumour growth impairment *in vivo*, due to loss of Aurora B stabilisation (Mallampalli, Glasser, Coon, & Chen, 2013). Collectively these data indicate that CaM has a complex, multi-faceted, role during mitosis and cytokinesis and highlight the importance of an EF hand containing Ca²⁺ binding protein during these essential cellular processes.

CaBP	Functions	References
Caldendrin	<ul style="list-style-type: none"> - Regulates Jacob (gene expression) - Binds to AKAP79/150 scaffolding protein - Modulates Cav1.2 (L-type) Ca²⁺channel 	(Dieterich et al., 2008; Gorny et al., 2012; Tippens & Lee, 2007)
CaBP1L and CaBP1S	<ul style="list-style-type: none"> - Regulates VGCC (P/Q- and L-type) - Regulates TRPC5 and IP3Rs 	(Haynes et al., 2004; Kinoshita-Kawada et al., 2005; A. Lee et al., 2002; C. Li et al., 2013; Lloyd-Evans et al., 2008; Oz et al., 2013)
CaBP2	<ul style="list-style-type: none"> - Regulates VGCC - Activates CaM kinase II 	(Haeseleer et al., 2000; Schrauwen et al., 2012)
CaBP3	N/A- pseudo gene	(Haeseleer et al., 2000)

CaBP4	<ul style="list-style-type: none"> - Regulates VGCC - Interacts with photoreceptor protein MRG4 	(Haeseleer, 2008; Haeseleer et al., 2000; 2004; Khan, 2014; S. Park, Li, Haeseleer, Palczewski, & Ames, 2014)
CaBP5	<ul style="list-style-type: none"> - Regulates VGCC in retina - Interaction with Munc-18 (SNARE complex protein) - Regulation of synaptic fusion 	(Rieke et al., 2008; Sokal & Haeseleer, 2011)
CaBP7	<ul style="list-style-type: none"> - PI4KIIIβ regulation - Role in cell division 	(McCue et al., 2012; Mikhaylova et al., 2009; Neumann et al., 2010)
CaBP8	<ul style="list-style-type: none"> - PI4KIIIβ regulation - Regulating N-, L-, P/Q- type channels on the PM 	(Mikhaylova et al., 2009; Shih et al., 2009)

Table 1.1. Summary of the CaBP protein family and their known functions.

Note* - For unknown reasons CaBP6 was not assigned as an identifier during the early characterization of the CaBP family.

1.2. The cell cycle

The mitotic cell cycle is an essential process for the development, growth, repair and normal aging of complex multicellular organisms. It comprises a series of events whereby a single cell duplicates its chromosome number and all of its genetic content to be ultimately apportioned into clonal daughter cells. This process has been studied since 1880, when for the first time the German scientist Walther Flemming noticed and described the nucleus' transformation prior to cell division as "threads" (Paweletz, 2001). Even though the process of cell division has been recognised for well over a century, advances in modern light microscopes were required to accurately

distinguish between various cell division stages and gain further insights into the distinct steps. The majority of the cell cycle comprises interphase, which can be further divided into Gap 1 (G1), Synthesis (S) and Gap 2 (G2) (Mitchison & Salmon, 2001). G1 is the preparation phase for DNA synthesis; here the cell functions normally however it will increase its normal size to allow for duplication of its DNA. S phase proceeds G1 phase, whereby the cell duplicates its chromosome number. This is followed by G2 where the cell continues growing in size in preparation for cell division. Genome replication and separation is the key event during division. Duplication occurs at S phase and segregation during Mitosis (M) phase (Diagram 1.5.) (Norbury & Nurse, 1992). Cell cycle progression is controlled by specific proteins, which regulate the onset of each phase in the correct sequence, to ensure the exact duplication of genetic material and its division into two new daughter cells. Cyclin- dependent kinases (Cdks) are the key enzymes controlling transitions through cell division (Sullivan & Morgan, 2007). Cdk activity requires as its name suggests cyclin protein binding, which are present at specific stages of the cell cycle. There are 4 Cdks directly involved in the mammalian cell cycle. Cdk 4/6 are controlled by D-type cyclins, which are active at the start of G1 phase, partly during G1/S progression and which regulate cell proliferation (Sheppard & McArthur, 2013). Subsequently, Cdk2 associates with A and E-type cyclins and this complex is the major regulator of G1/S and S/G2 transition (Nasmyth, 1996). Active Cdk2 phosphorylates critical proteins involved in DNA replication, histone synthesis and centrosome duplication (Krasinska et al., 2008; Meraldi, Lukas, Fry, Bartek, & Nigg, 1999).

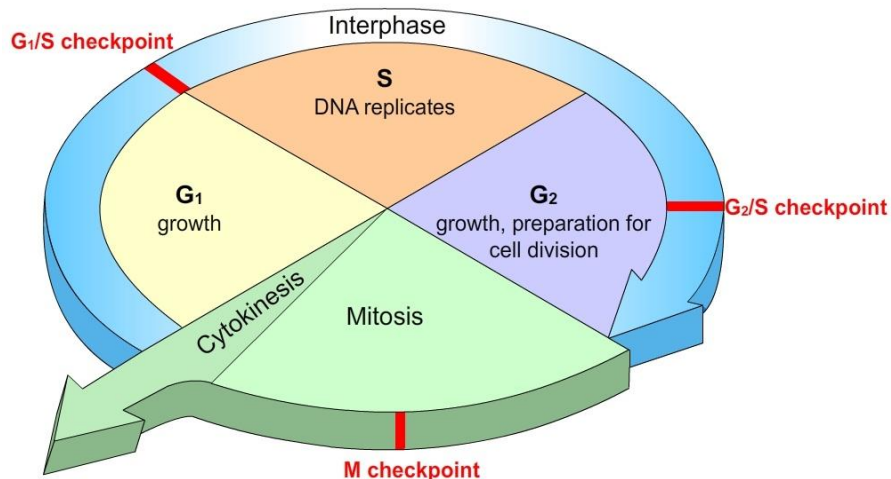


Diagram 1.5. Cell cycle divided into five phases. Each phase is illustrated in a different colour and labelled with the main process involved. An arrow indicating exit from the cycle at cytokinesis is for cells which no longer duplicate and after cytokinesis enter a phase known as G₀ or quiescence such as neurons (Adapted from: RicochetScience. 2015.) Available at: <http://ricochetscience.com/brca1-cancer/>. [Accessed 05 August 15].

One such critical target for sequential phosphorylation by cyclin D/Cdk4 and cyclin E/Cdk2 complexes is the tumour suppressor protein retinoblastoma (pRb). In its hyper-phosphorylated state pRb activates E2F family transcription factors to regulate expression of genes required for completion of S phase. Nuclear kinase WEE1 and checkpoint kinase 1 (Chk1) impede Cdk1 function via phosphorylation (Fasulo et al., 2012) until DNA replication is complete. Once replication is accomplished, Cdk1 phosphorylates and inactivates nuclear kinase WEE1 and Cdk1 itself becomes active. Cdk1 then binds to cyclin A2 and B1 and phosphorylates critical proteins controlling nuclear envelop breakdown, chromosome condensation and mitotic spindle organization.

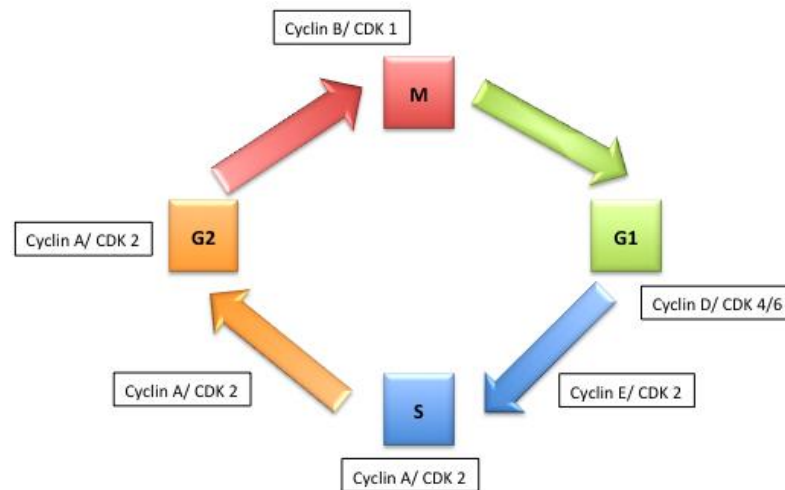


Diagram 1.6. Cell cycle progression regulated by Cdk1 and cyclins. Each phase is illustrated in a different colour and the involved Cdk1 – cyclin complexes responsible for cell cycle progression at various checkpoints are listed in boxes accordingly.

Hence Cdk1 is responsible for the progression from G2- M phase (Takizawa & Morgan, 2000). In summary, progression through interphase is dependent on the orchestrated and fluctuating synthesis and degradation of specific cyclins and their complex formation with the appropriate Cdk1 which act as critical checkpoints at each stage (Diagram 1.6.) (Nurse, 2002; Vermeulen, Van Bockstaele, & Berneman, 2003). Cdk1 are considered master regulators of the cell cycle however there are various other signalling cascades that also modulate this process. Mitosis is the final phase of the cell cycle where replicated chromosome segregation occurs. This process can be further divided into 5 sub-phases based on chromosomal localisation and state of the mitotic spindle, these are: 1) Prophase, 2) prometaphase, 3) metaphase, 4) anaphase and 5) telophase (Mitchison & Salmon, 2001), followed by cytokinesis. The main events during prophase/prometaphase are nuclear envelope break down and condensation of replicated chromatin into

chromosomes. During metaphase, spindle fibres align all chromosomes along the mitotic plate and attach them to the microtubules and the centromere. These spindle fibres shorten, pulling the centrosomes to opposite spindle poles and chromatids travel along the microtubules to each pole at anaphase. At telophase the chromosomes have arrived at the opposite poles, the spindle fibres disappear and the nuclear envelope reforms around the new DNA pools. Cells are now fully equipped for physical separation, which finally occurs during cytokinesis (Diagram 1.7.) (Leman & Noguchi, 2014). Cytokinesis is often regarded as a separate stage of the cell cycle as the events that occur there are geared toward the physical separation of the mother cell into the two new daughters in contrast to mitosis which focuses on the accurate segregation of duplicated genetic material. Collectively these stages mediate the precise division of a single mother cell into two duplicate daughter cells. The sequential stages allow for tight regulation and strict quality control, which prevent genetic errors and abnormalities from being transmitted to the new cells. Tight regulation, synchronization and activity of hundreds of proteins are required for successful completion of cell division.

1.2.1. Importance of cytokinesis

Cytokinesis derives from the Greek words cyto = cell and kinesis = movement. As the translation suggests this is a dynamic process, which occurs at the final stage of mitosis as mentioned above. It is the physical separation of the cells, whereby the cytoplasm is apportioned exactly into duplicate daughter cells.

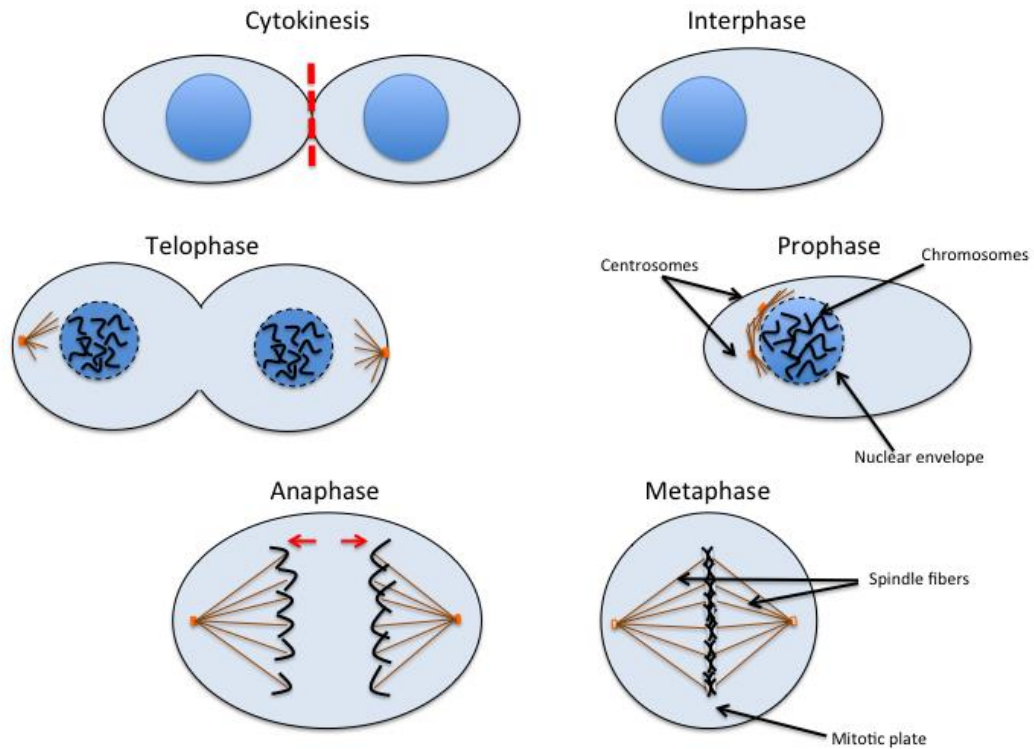


Diagram 1.7. Simplified overview of mitosis. Mitosis is the final stage of the cell division and can be further divided into sub- phases. These different stages are illustrated here including prophase, metaphase, anaphase, telophase and finally cytokinesis.

This step is highly regulated and coordinated in space and time to ensure that each cell obtains the correct quantity of DNA and other cellular materials/organelles. Cytokinesis in all eukaryotes is reliant on the cytoskeletal proteins tubulin and actin in addition to other key factors. The importance of cytokinesis cannot be underestimated. Failure can result in unstable tetraploid cells harbouring abnormal numbers of centrosomes (Fujiwara et al., 2005). Additionally, cells that cannot segregate their chromosomes correctly, can potentially become aneuploid having altered growth properties that can promote tumorigenesis (Lv et al., 2012). The link

between aberrant cytokinesis and cellular transformation is highlighted in studies demonstrating that mutations in tumour suppressor genes result in an increased frequency of cytokinesis failure (Caldwell, Green, & Kaplan, 2007; Ciapa, Pesando, Wilding, & Whitaker, 1994; Whitaker, 1997; 2008). Additionally, components required for cytokinesis are often up regulated in tumours or lie in regions of chromosomes either deleted or amplified in tumours (Corson and Gallie, 2006; Roversi, 2006). Therefore it is of fundamental importance to completely understand the activity of proteins (including CaBP7) and the underlying molecular mechanisms involved in cytokinesis under normal physiological conditions. This knowledge will give a clearer understanding about how misregulation of these factors can lead to cytokinesis failure and potential tumorigenesis.

1.2.2. Molecular mechanism of cytokinesis

Cytokinesis can also be further divided into defined- stages, as it is a complex multi-step process. A comprehensive overview of cytokinesis can be found in Barr and Gruneberg's review and an updated review from Green *et al.* (Barr & Gruneberg, 2007; Green, Paluch, & Oegema, 2012). Here, the essential steps and proteins involved in cytokinesis are summarised. The first step of cytokinesis is to specify the division plane; this requires precision to ensure equal division of material between the cells. Microtubules play a key role in selecting the site of division. The central spindle selects the division site, by physically interacting with the cell cortex (a specialised cytoplasmic protein inner layer) prior to furrow formation and ingression (Barr & Gruneberg, 2007; Glotzer, 2005). The central spindle establishes during

anaphase, the step prior to cytokinesis and is the region where anti-parallel microtubules overlap in the centre of the spindle after the chromosomes have moved to opposite poles. At the establishment of a nascent equatorial zone, active RhoA GTPase accumulates (Nishimura & Yonemura, 2006). Cleavage furrow formation follows where RhoA plays a crucial role to regulate actin dynamics (Piekny, Werner, & Glotzer, 2005). RhoA accumulates in a narrow zone on the equatorial cortex at the site selected for the cleavage furrow (Bement, Benink, & Dassow, 2005; Piekny et al., 2005). RhoA depletion or biochemical inactivation leads to furrow ingression failure in telophase. RhoA cycles between active GTP-bound and inactive GDP-bound form, which is regulated by the guanine exchange factor (GEF) epithelial cell-transforming 2 (Ect2). RhoA-GTP primarily activates crucial contractile ring components including formin (for actin polymerization activation), Rho dependent kinases (ROCK, regulates myosin that is essential for contractile ring dynamics) and Citron kinase (Citron K, which activates myosin II), but additionally also activates effectors such as F-actin and anillin at the cleavage furrow (Diagram 1.8.) (Jaffe & Hall, 2005; Piekny et al., 2005).

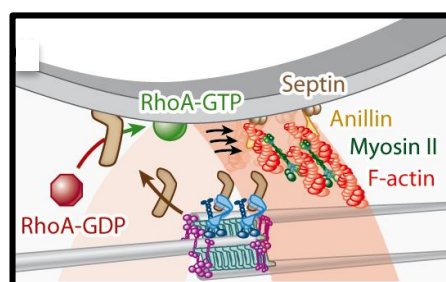


Diagram 1.8. Detailed schematic of furrow ingression machinery. RhoA becomes active upon GTP binding, which in turn leads to activation and assembly of key components of the contractile ring machinery (Green et al., 2012).

During early cleavage furrowing two serine/threonine kinases are also implicated: Aurora B kinase and Polo-like kinase 1 (Plk1). Plk1 phosphorylates the mitotic kinesin like protein 2 (Mklp2), which is involved in localising other important components during cytokinesis (Neef et al., 2007). Aurora B is part of a larger complex with inner centromere protein (INCENP), inhibitor of apoptosis (IAP), repeat protein Survivin and Borealin which collectively form the chromosomal passenger complex (CPC). The CPC associates with chromatin during early mitosis and accumulates at the centromere in prometaphase and metaphase (Carmena, Wheelock, Funabiki, & Earnshaw, 2012). At the onset of anaphase the CPC is transferred from the centromeres to the spindle midzone enabled by Mklp2 mitotic kinesins (Mishima, Pavicic, Gruneberg, Nigg, & Glotzer, 2004). Cytokinesis can fail when this anaphase checkpoint is disrupted (Gruneberg, Neef, Honda, Nigg, & Barr, 2004). Studies have shown CPC and Aurora B to promote and to be essential for cleavage furrow induction and ingression (Furuya et al., 2010; Gruneberg et al., 2004). The CPC in particular recruits centralspindlin to the spindle midzone. The mitotic kinesin Mklp1 and the GTPase-activating protein MgcRacGAP are part of the centralspindlin complex linking Rho regulation at the cell cortex with the central spindle microtubules (Mishima, Kaitna, & Glotzer, 2002). This complex can then assemble with Ect2, which allows a ternary complex linking the central spindle microtubules with the cell cortex to spatially restrict Rho activation and actin polymerization (Barr & Gruneberg, 2007). Besides protein based links between centralspindlin and the cell surface, recent data has been published showing the function of phosphatidylinositol lipids such as PI (4,5)

P₂ during furrow positioning and cytokinesis (Janetopoulos & Devreotes, 2006; R. Wong et al., 2005). Studies have demonstrated an accumulation of PI (4,5) P₂ in the cleavage furrow and a requirement for lipid kinases at cytokinesis (Brill, Wong, & Wilde, 2011). Although the importance of RhoA activation and actin polymerization has been studied during furrow formation exactly how the constriction of the contractile ring occurs remains to be fully elucidated. Investigations have shown that actin and myosin II accumulation at the cell equator lead to the formation of contractile filaments and are drawn together in a “purse – string” like mechanism by the action of myosin II (Barr & Gruneberg, 2007). Septin assembly is always observed in two spatially restricted rings at the cell cortex that flank either side of the acto-myosin contractile ring. This highly specific localisation is directed by anillin and provides stability and restriction of the cleavage furrow (Diagram 1.9.) (Barr & Gruneberg, 2007).

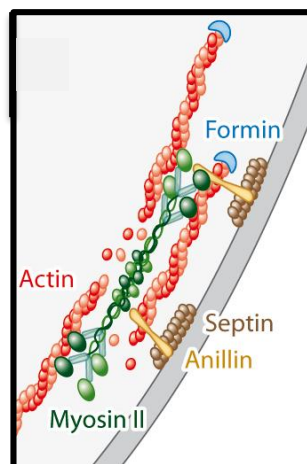


Diagram 1.9. Detailed schematic of contractile ring constriction. The contractile ring network is composed of formin bound- actin filaments and myosin II which is linked to membrane associated- septins via filamentous cross linker septins (Green et al., 2012).

The purse- string closure mechanism allows the contractile ring to encircle the central spindle and eventually anillin crosslinks actin, membrane and septin filaments. The final step required to complete division is the process of abscission resulting in two physically separate daughter cells. It is known that membrane trafficking and fusion events are essential for normal abscission. Abscission is a complex, tightly regulated process with various molecular components playing a role in severing the intercellular bridge to release new daughter cells (Steigemann & Gerlich, 2009). The most widely studied and core complex involved in cell separation is the endosomal-sorting complex required for transport III (ESCRT-III). Cep-55 is a midbody protein, which recruits ESCRT-III via its interaction with ALG-2 interacting protein X (ALIX) and tumour susceptibility gene 101 (Tsg 101) (Diagram 1.10.). However Cep-55 localisation to the midbody is in turn dependent on Mklp1 kinesin (Carmena, 2012). Studies investigating the onset and trigger for abscission revealed that pulling tension release initiates ESCRT-III assembly and eventual fission (Lafaurie-Janvore, Maiuri, 2013).

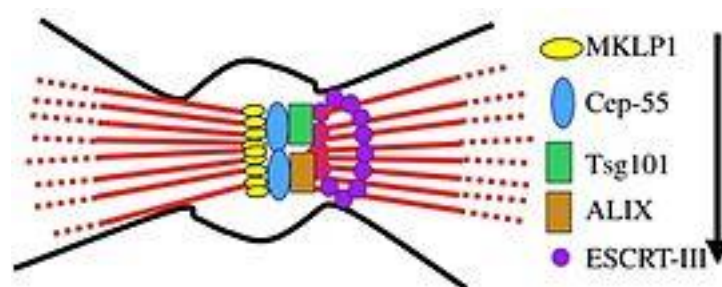


Diagram 1.10. Simplified view of molecular machinery at the midbody prior to abscission. Plk1 degradation induces dephosphorylation and activation of Cep55 and marks the onset of abscission initiation, Cep55 binds to Mklp1 and recruits Tsg101 and ALIX, which stimulates ESCRT- III recruitment facilitating scission (Carmena, 2012).

Once abscission is completed the ESCRT-III complex is degraded in an energy- consuming process by vacuolar protein sorting-associated protein 4 (Vps4) (Henne, Buchkovich, & Emr, 2011). Abscission leads to the shearing of the plasma membrane therefore insertion of additional membrane from an internal reservoir is essential at this point to prevent leakage of cell contents. The octameric exocyst protein complex consisting of Sec-3, -5, -6, -8, -10, -15, Exo70 and Exo84 is involved in vesicle trafficking and especially in targeting of post-Golgi vesicles to the plasma membrane for membrane insertion and donation (Boucrot & Kirchhausen, 2007; Munson & Novick, 2006). The exocyst is targeted to the cleavage furrow via its interaction with RalA, Rab11 and the Rab11 effector protein FIP3 (Chen et al., 2006, Fielding et al., 2005, Wilson et al., 2005). Endosomes and Golgi have been observed to cluster around the centrosomes but to be excluded from the midbody and endosomal fusion has been reported to be important for successful abscission (Boucrot & Kirchhausen, 2007; Hill, Clarke, & Barr, 2000). Interestingly, lysosomes also cluster at centrosomes and to either side of the intercellular bridge and are excluded from the midbody although their function during cytokinesis remains unknown (MATTEONI & KREIS, 1987). A further intriguing observation is that CaBP7 depletion in HeLa cells also appears to exert functional consequences during late stages of cytokinesis with cells unable to perform normal abscission (Neumann et al., 2010). Cytokinesis is a very intricate process involving interactions between many proteins and protein complexes and only the essential details are described here. During cytokinesis extensive membrane trafficking is required for increasing the cell surface, therefore a vast amount of plasma membrane

lipid remodelling occurs (Echard, 2012; Emoto, Inadome, Kanaho, Narumiya, & Umeda, 2005).

Protein	Function	Reference
Rho A	Regulates effector proteins in the actomyosin contractile network	(Piekny et al., 2005)
Aurora B	Targets proteins to cleavage furrow and part of CPC	(Carmena, 2012)
F-actin and myosin II	Allow contractile ring ingression	(Green et al., 2012)
Polo- like kinase 1 (Plk)	Phosphorylate and activates kinesin Mklp2	(Kraut, Peters, Glotzer, 2007)
CPC	Regulates contractile ring constriction and maturation, recruits centralspindlin to midzone	(Carmena et al., 2012)
Septins	Part of the contractile ring	Maddox et al., 2007
Anillin	Crosslinks septins, F-actin and myosin in contractile ring	Maddox et al., 2007
ALIX, Tsg101 ESCRT-III	Completes abscission	(Caballe & Martin-Serrano, 2011)

Table 1.2. Key proteins involved in cytokinesis and their documented molecular functions.

1.2.3. Regulation of cytokinesis

The temporal onset of cytokinesis is as tightly regulated as the other mitotic phases that precede it. The beginning of anaphase occurs upon Cdk1/ cyclin B down regulation. This is a strict requirement since abnormal, persistent,

activation of this complex leads to disruption of the central spindle assembly, prevention of contractile ring formation and ultimately cytokinesis failure (Green et al., 2012; Wheatley et al., 1997). In agreement with these observations Cdk1 inhibition with specific drugs is sufficient to induce cytokinesis even in the absence of chromosome segregation (Niiya, Xie, Lee, Inoue, & Miki, 2005). Therefore Cdk1/cyclin B activity negatively regulates protein complexes required for cytokinesis. In addition, Cdk1 exerts another level of regulation by phosphorylating and inhibiting the kinesin motor Mlkp1 and inner centrosome protein (INCEP), which is part of the CPC. Inactivation of Mlkp1 prevents interaction with and repositioning of the centralspindlin complex (Mishima et al., 2004). In contrast to Cdk1, Aurora B and Polo kinase activities promote cytokinesis (Gruneberg et al., 2004; Vader, Medema, & Lens, 2006).

Further unique temporal regulation occurs through sequestration of components required for cytokinesis to the nucleus. For example, the RhoGEF Ect2 contains two nuclear localisation signals (NLSs) and is predominantly nuclear localised to prevent premature activation of RhoA which is only found in the cytosol (Tatsumoto, Xie, Blumenthal, Okamoto, & Miki, 1999; Yüce, Piekny, & Glotzer, 2005). Similarly, mitotic kinesins and spindle microtubule associated proteins (MAPs) responsible for remodelling the microtubule cytoskeleton at the onset of anaphase are also stored in the nucleus (Green et al., 2012; Neef, Klein, Kopajtich, & Barr, 2006) and remain inactive until nuclear envelope break down occurs. In later stages of cytokinesis (when the nuclear envelope has reformed) the re-uptake of proteins into the nucleus is also an essential regulatory step. Ect2 is

trafficked back into the nucleus upon nuclear envelope reformation, as its persistent cytosolic activity might limit furrow ingression and therefore obstruct the actin polymerisation required for adjacent membranes to reach sufficiently close proximity for abscission to occur. Other molecules such as the Mklp1, which is essential for midbody integrity, also exhibit an additional level of regulation. The nuclear localisation sequence of Mklp1 is phosphorylated by Aurora B thus preventing its re-entry into the nucleus until cytokinesis completion (Neef et al., 2006).

Regulation also occurs through protein degradation mechanisms. The machinery responsible for proteolytic inactivation during mitosis and cytokinesis is the anaphase- promoting complex/cyclosome (APC/C) cullin-RING finger E3 ubiquitin ligase. This ubiquitin ligase recognises target substrates via Cdc20 and Cdh1 adaptor proteins (Peters, 2006). Critical regulation in cytokinesis by APC/C occurs by reducing Cdk1/cyclin B activity (as described above) (Pines, 2011). Plk1 is also degraded during anaphase by the APC/C and this permits abscission initiation by recruitment of Cep55 to the midbody (Bastos & Barr, 2010). APC/C is considered a master regulator as nearly all key components required for successful cytokinesis are targeted for degradation at defined temporal intervals by the APC/C (Green et al., 2012). In conclusion the regulation of cytokinesis exhibits complex multi-level control. A spatial component of the regulatory system exists whereby molecules involved in cytokinesis are stored in the nucleus and only activated once freed upon nuclear envelope breakdown. A specific temporal regulatory mechanism also operates in early mitosis when Cdk1 phosphorylation inhibits selected proteins, which could otherwise promote

premature cytokinesis. At anaphase these inhibitory modifications are reversed and phosphorylation of Aurora B and Polo like family kinases in turn permit the cytokinesis machinery to form to ensure cytokinesis completion. In addition, proteolysis of proteins required for cytokinesis by the APC/C permits a third layer of regulation through selective protein inactivation.

1.3. Phosphatidylinositides

Phosphatidylinositides (PIs) typically compose ~ 1% of total cellular phospholipids, however they represent an extremely diverse lipid family. The parent lipid, phosphatidylinositol (PI, accounts for ~15% of total cellular phospholipid in most eukaryotes), can give rise to seven possible PIs by phosphorylation at the myo-inositol head group at position 3, 4 and 5 (Diagram 1.11.). The hydrophilic head group of PIs is exposed to the cytosol and anchored to membranes by insertion of two acyl chains esterified to a glycerol moiety (T. Balla, 2013). These lipids were first discovered in the 1970s and ever since their characterised range of biological functions have increased (T. Balla, 2013). PI is synthesised in the ER from cytidine diphosphate- diacylglycerol (CDP-DAG) and myo-inositol by PI synthase enzymes and then distributed to its ultimate destination by PI transfer proteins or vesicular trafficking (Agranoff, Bradley, & Brady, 1958; Sha & Luo, 1999). PIs are specifically localised and are key determinants of membrane compartment identity (Diagram 1.12.). Due to their pleiotropic functions, PIs have been implicated in cellular roles including cell growth, survival, architecture, signalling, ion channel/ pump regulation, membrane

trafficking, endocytic and exocytic processes and cytoskeleton dynamics (T. Balla, 2013). Misregulation of PI metabolism can lead to diseases such as diabetes, obesity and cancer (T. Balla, 2013; Pendaries, Tronchère, Plantavid, & Payraastre, 2003). More recent research has revealed that the PI regulatory system is also essential for the infective capacity of certain human viruses including Hepatitis C virus (HCV). Viruses of this type hijack components of the PI signalling machinery to generate a replication complex (RC) for the virus' RNA (C. Lee, 2012). HCV specifically hijacks host cell enzymes to create a phosphatidylinositol 4-phosphate (PI4P) rich area at the membrane, which serves as a viral replication platform (Bishé, Syed, & Siddiqui, 2012b; Delang, Paeshuyse, & Neyts, 2012). PI4P and phosphatidylinositol 4,5-bisphosphate (PI (4,5) P₂) are the most abundant PIs in the cell.

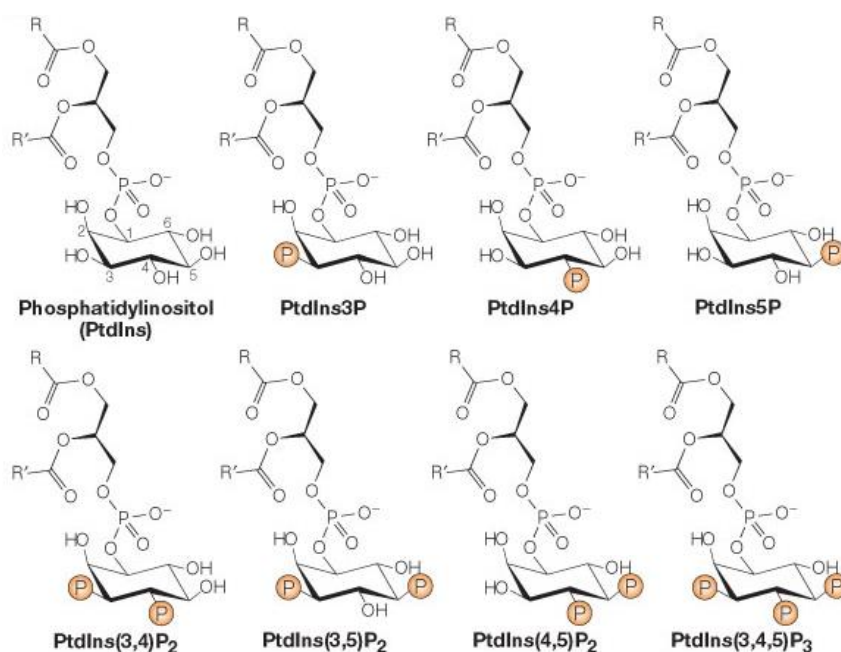


Diagram 1.11. Illustration of Phosphatidylinositol (PIs). Phosphatidylinositol and its seven possible phosphorylated derivatives are shown. Phosphate groups are depicted in orange (Le Roy & Wrana, 2005; Pu et al., 2015).

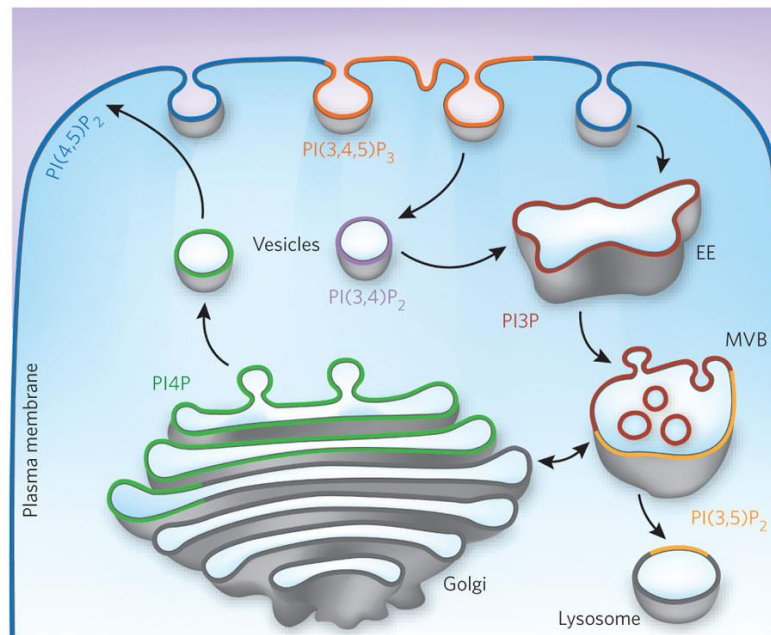


Diagram 1.12. The cellular distribution of phosphoinositide lipids. Each phosphoinositide lipid is depicted in a different colour and its predominant localisation within the cell is illustrated (Kutateladze, 2010).

The appearance and distribution of these two PIs depends on the activity of specific kinases and phosphatases. PI4P has been long believed to only act as the major precursor for PI (4,5) P₂, however it is now recognised that PI4P exerts unique signalling functions of its own. PI4P is an established and important regulator of membrane trafficking in yeasts, plants, insects and mammals (De Matteis, Godi, & Corda, 2002). PI4P has recognised Golgi functions including protein sorting, vesicular carrier biogenesis, protein transport from the TGN to the PM and in the normal localisation of Golgi resident proteins (De Matteis, Wilson, & D'Angelo, 2013). PI4P has also Golgi- independent roles and is involved in endosomal trafficking, ER export, autophagy, signalling at the PM, cytokinesis and actin dynamics (De Matteis et al., 2013; Tan & Brill, 2014). Recently, a novel role for PI4P has been identified on lysosomes, where it regulates lysosomal content sorting

(Sridhar et al., 2013). The production of PI4P is facilitated by four phosphatidylinositol 4-kinases (PI4Ks) which phosphorylate PI to produce PI4P, which in turn can be further phosphorylated by either phosphoinositide 3-kinase (PI3K) or phosphatidylinositol 4-Phosphate 5-Kinase (PIP5K) generating phosphatidylinositol 3,4-bisphosphate (PI (3,4) P₂) or PI (4,5) P₂ respectively (Diagram 1.13.). PI (3,4) P₂ and PI (4,5) P₂ can also be hydrolysed back to PI4P by the activities of phosphatidylinositol-3-phosphatase (PI3P) or phosphatidylinositol-5-phosphatase (PI5P) respectively. PI4P can also be dephosphorylated to PI by the action of the phosphatidylinositol phosphatase Sac1 (Tan & Brill, 2014).

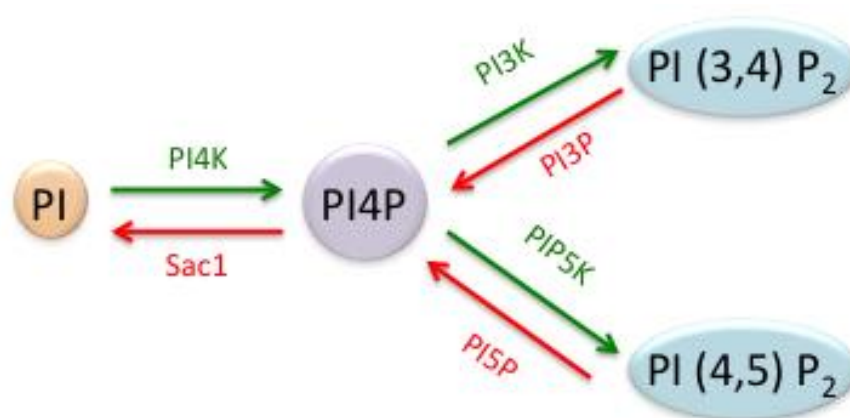


Diagram 1.13. Generation and interconversion of PI4P. Green arrows and enzymes indicate kinases that add a phosphate group and red arrows and enzymes denote phosphatases, which remove a phosphate group. **PI** = Phosphatidylinositol, **PI4P** = Phosphatidylinositol 4-phosphate, **PI (4,5) P₂** = Phosphatidylinositol 4,5-bisphosphate, **PI (3,4) P₂** = Phosphatidylinositol 3,4-bisphosphate, **PI4K** = Phosphatidylinositol 4-kinases, **PI3K** = Phosphatidylinositol-4-phosphate 3-kinase, **PIP5K** = Phosphatidylinositol 4-Phosphate 5-Kinase, **PI3P** = Phosphatidylinositol-3-phosphatase, **PI5P** = phosphatidylinositol-5-phosphatase.

1.3.1. PIs and Cytokinesis

Studies in sea urchin embryos have revealed insights into phosphoinositide metabolism during mitosis and cytokinesis (Baccetti and Whitaker, 1997). Abnormal PI levels have been associated with cytokinesis defects and failure in particular altering furrow stability and final abscission (Echard, 2012). The main PI species involved at cytokinesis are PI3P, PI4P, PI (4,5) P₂ and PI (3,4,5,) P₃.

In this thesis PI4P is the lipid of interest during cytokinesis. PI4P is generated from the enzymatic activity of PI4Ks. In three different species of yeast (Garcia-Bustos, Marini, Stevenson, Frei, & Hall, 1994), a protozoan (*Trypanosome brucei*) (Rodgers, Albanesi, & Phillips, 2007) and *drosophila* (Polevoy et al., 2009) the specific activity of a single PI4K isoform, PI4KIII β , has been shown to be essential for successful cytokinesis. PI4KIII β mutant yeast strains exhibited a cytokinesis failure with all mutant cells appearing in pairs with fully separated nuclei (Garcia-Bustos et al., 1994). *T.brucei* cells depleted of PI4KIII β could not complete division, again due to a cytokinesis block (Rodgers et al., 2007). In *drosophila*, *four wheel drive* (*fwd*) mutant flies (the *fwd* gene encodes the fly PI4KIII β orthologue) cells exhibited unstable intercellular bridges and disorganised contractile ring F-actin leading to a multinucleate phenotype. Collectively, these findings strongly suggest that PI4KIII β regulation and its product PI4P play key roles during cell division especially at cytokinesis.

PI4P is the principal precursor for PI (4,5) P₂ and the role of this PI has also been studied during cytokinesis. PI (4,5) P₂ has established roles in endocytosis and exocytosis (Mayinger, 2012), and both processes are active

during cytokinesis (Neto, 2011). At late furrow ingression, PI (4,5) P₂ directly recruits the exocyst complex to the intercellular bridge, which is essential for abscission (Echard, 2012). PI (4,5) P₂ levels are tightly regulated throughout cytokinesis to successfully complete separation. PI (4,5) P₂ accumulation at the cytokinesis furrow is required for furrow ingression and intercellular bridge stability. Anillin, a key player in membrane furrowing, interacts with PI (4,5) P₂ for its correct localisation, which facilitates correct furrow organisation (Liu, et al., 2012). It has also been observed that septin 2 cannot localise to the furrow if PI (4,5) P₂ is depleted from this region. Septins are important GTP-binding proteins involved in intercellular bridge stability (Estey, Di Ciano-Oliveira, Froese, Bejide, & Trimble, 2010; Kinoshita et al., 1997). Although the examples aforementioned highlight that PI (4,5) P₂ is required for normal cytokinesis, it appears that hydrolysis of this PI is also essential and that dynamic creation and turnover is required at discrete stages of cytokinesis. OCRL (the inositol 5-phosphatase) localises to the midbody at cytokinesis and its depletion or inhibition (thereby increasing PI (4,5) P₂ levels) has been linked to the formation of stable intercellular bridges, having, as expected, increased levels of PI (4,5) P₂, and resulting in significant delays in abscission. This data demonstrates that depletion of this lipid is required to create suitably labile intercellular bridges (Dambournet et al., 2011). This defect could stem from observations that prevention of PI (4,5) P₂ turnover induces F-actin enrichment and corresponding abscission defects (Moulding et al., 2012). Therefore temporal production and hydrolysis of PI (4,5) P₂ are both essential during cytokinesis (Echard, 2012).

1.3.2. Phosphatidylinositol 4-kinase III beta (PI4KIII β)

There are four phosphatidylinositol 4-kinases (PI4Ks) divided into 2 classes (II and III) primarily based on their sequence similarity and sensitivity towards non-specific inhibitors such as wortmannin and adenosine. Type II kinases are sensitive to adenosine, whereas type III activity is inhibited by wortmannin (A. Balla & Balla, 2006). All four enzymes phosphorylate PI at the D4 inositol head-group position to produce PI4P, an essential initial step in the enzymatic pathways generating PI (4,5) P₂, PI (3,4) P₂ and other PI signalling molecules. PI4KII α and PI4KIII β are predominantly Golgi targeted, whereas PI4KIII α is mainly PM associated and PI4KII β can be seen in the cytosol and associated with trafficking vesicles (Clayton, 2012). Recently a biochemically distinct pool of PI4KIII β has been identified on lysosomes (Sridhar et al., 2013). PI4P is produced at many cellular membranes but predominately those of the Golgi apparatus, where crucial transport related functions, as previously described, have been demonstrated (Hammond, 2012). There is limited PI4P at the PM since the majority is used for the synthesis of PI (4,5) P₂ (Hammond, 2012). In this thesis the main focus of phosphoinositide metabolism has been on TGN and lysosome resident PI4KIII β activity and regulation of PI4P levels during mitosis.

1.3.3. Regulation of PI4KIII β

PI4KIII β is localised to the Golgi by direct binding to ADP ribosylation factor - 1 (Arf1). Arf1 is a PI4KIII β activator and is therefore able to modulate Golgi PI4P levels (Godi, Pertile, Meyers, Marra, & Di Tullio, 1999). Golgi resident PI4KIII β is additionally activated at the TGN by NCS-1 in a Ca²⁺ dependent

manner (Haynes et al., 2005). NCS-1 and Arf1 antagonise one another's ability to stimulate PI4KIII β and this may represent a mechanism that permits the formation and maintenance of distinct Arf1 controlled and NCS-1 controlled trafficking domains on the TGN (Haynes et al 2005). More recently, CaBP7 and 8 have also been shown to negatively regulate PI4KIII β on the TGN (Mikhaylova et al., 2009), and it seems that there is a complex regulatory network consisting of Arf1, NCS-1 and CaBP7 and 8 which is required for precise spatio-temporal control of PI4KIII β activity (Burgoyne & Haynes, 2010). Tight control of PI4KIII β at the TGN seems appropriate due to the important role that it plays in controlling vesicle mediated export from the Golgi apparatus via its product PI4P (Bishé, Syed, Field, & Siddiqui, 2012a). PI4P is responsible for recruiting signalling molecules that contain PI4P binding motifs important for ER and Golgi transport and reduction of PI4P levels leads to reduced cargo trafficking from the TGN to PM and to endosomes (Bishé et al., 2012a; Szentpetery, Várnai, & Balla, 2010). One important example of a PI4P binding protein with functional consequences for Golgi trafficking is Golgi- phosphoprotein 3 (GOLPH3). Binding of GOLPH3 to PI4P and recruitment of unconventional myosin Myo18a generates the necessary tensile force to bud nascent transport vesicles from the TGN (Dippold et al., 2009; Tan & Brill, 2014).

An additional level of PI4KIII β regulation occurs via protein kinase D (PKD). PI4KIII β is phosphorylated and activated by PKD thereby creating a binding site on PI4KIII β for 14-3-3 proteins which stabilize the active conformation of the enzyme (Hausser et al., 2006; 2005).

Additionally Sridhar *et al.* identified a biochemically distinct lysosome resident pool of PI4KIII β which dynamically associates with lysosomes and influences lysosomal content sorting (Sridhar *et al.*, 2013). Results from their study demonstrate the importance of PI4P produced by lysosomal resident PI4KIII β in regulating vesicular carrier budding and fission from lysosomes, a function consistent with the documented role of Golgi associated PI4KIII β .

1.4. Lysosomes

Lysosomes are membrane bound organelles first discovered in 1949 by the Belgian cytologist Christian Rene de Duve and which have subsequently been observed in many eukaryotic cell types. Lysosomes are highly distinctive in terms of their molecular make-up and show some variation in morphology having a heterogeneous distribution of diameters ranging between 0.2-1.2 μm (De Duve & Wattiaux, 1966). Lysosomes have a cholesterol poor lipid bilayer harbouring numerous types of membrane protein. These membrane proteins are required for transport of substrates into and out of the lysosomal lumen. Furthermore there are a subset of specialised membrane proteins (small Rab GTPases and SNAREs) that mediate lysosomal trafficking and fusion processes (Saftig & Klumperman, 2009). More extraordinary is the acidic lumen of lysosomes, which is maintained at a pH of 4.6 – 5.0, with the surrounding cytosol at ~pH 7.2 (Mindell, 2012). This acidity is generated by the activity of the vacuolar ATPase (V-ATPase), which moves (H^+) protons against their concentration gradient in to the lysosome lumen. The V-ATPase is a highly evolutionarily

conserved enzyme, which operates on endosome, lysosome and secretory vesicle membranes (Forgac, 2007). The acidic environment of the lysosome lumen is essential for the catalytic activity of specialised degradative enzymes. Current data indicates there to be approximately 60 different lysosomal hydrolases (Settembre, Fraldi, Medina, & Ballabio, 2013; Xu & Ren, 2015). Hydrolases include sulphatases, glycosidases, peptidases, phosphatases, lipases and nucleases, which all have specific target substrates. These dedicated enzymes do not function or have very weak activities at cytoplasmic pH, which represents a highly evolved method to protect the cell from digestion and destruction in the event of lysosomal leakage. Additionally, a glycocalyx (glycoprotein-polysaccharide layer) lines the internal membrane of the lysosome to shield the lysosome membrane and proteins associated with or resident therein from degradation (Schwake, Schröder, & Saftig, 2013). Lysosomal resident proteins are divided into two broad classes: the soluble lysosomal hydrolases (acid hydrolases, ~60 unique enzymes in mammals), responsible for degradation of cellular macromolecules, and the integral membrane proteins (LMPs) which assist in degradative processes by ensuring acidification of the lysosome lumen in addition to transporting substrates for degradation (Appelqvist, Wäster, Kågedal, & Öllinger, 2013). LMPs exert additional important lysosome specific functions including control of membrane fusion events and the transport of recycled catabolised materials back to the cytoplasm (Saftig & Klumperman, 2009). Membrane fusion mediated by LMPs can also be important for degradative function if target substrates are being delivered to the lysosome by autophagosomes (discussed in detail in section 1.4.2.2).

1.4.1. Lysosomal hydrolases and membrane proteins

The most abundant Lysosomal membrane proteins (LMPs) are Lysosomal associated membrane proteins 1 and 2 (LAMP1, LAMP2), Lysosomal integral membrane protein 1 (LIMP1) and CD63, that are involved in many essential functions such as autophagy, membrane repair and virus infection (Saftig & Klumperman, 2009; Schwake et al., 2013). Additionally lysosomes contain over ~60 soluble hydrolases that can be divided into 3 subgroups depending on the amino acids in the catalytic dyad (aspartate, cysteine and serine cathepsins). These target specific substrates for degradation or bulk degradation and are additionally involved in antigen presentation and initiation of apoptosis (Luzio, Pryor, & Bright, 2007).

Lysosomes are the primary catabolic compartments of most eukaryotic cells, and this activity relies on functional hydrolases and LMPs. It is therefore important to understand how lysosome resident proteins reach this organelle. Acid hydrolases are delivered to the lysosomes on a direct route whereas LMPs can be delivered either directly from the TGN or arrive at the lysosome by an indirect route that involves initial transport to the PM and subsequent retrieval through internalisation and entry into the endocytic pathway (Saftig & Klumperman, 2009).

All lysosomal hydrolases are delivered to lysosomes by a direct transport mechanism involving the mannose- 6 phosphate (M6P) receptor (M6PR). This relies on the presence of a M6P tag in the oligosaccharide groups attached to the protein, which is recognised by TGN resident M6PR (Eskelinen, Tanaka, & Saftig, 2003). There are two types of M6PRs: the 300 kDa cation- independent M6PR (CI-M6PR) and the smaller 46 kDa cation-

dependent M6PR (CD-M6PR) (Braulke & Bonifacino, 2009). As their names suggest, the large receptor isoform binds to M6P independently of cations in contrast to the smaller variant, which requires cation binding. Both receptors are involved in trafficking acid hydrolases to lysosomes, however CI-M6PR is a multifunctional receptor implicated in many other cellular functions such as cell growth and motility (Ghosh, Dahms, & Kornfeld, 2003). In the TGN M6PRs binds to the Adaptor protein 1 (AP1) complex and/or Golgi- localised, gamma-ear containing ADP-ribosylation factor binding protein (GGA) to induce formation of a clathrin coated vesicle (60-100 nm), which can then travel through the direct pathway to endosomes. Arrival at the mildly acidic endosomal compartment causes M6PRs to dissociate from their acid hydrolase ligands. They are then recycled back to the TGN for the next round of transport by vesicular transport (Ghosh et al., 2003; Saftig & Klumperman, 2009). The acid hydrolases now in the endosome lumen are eventually delivered to lysosomes through dynamic fusion-fission events between early and late endosomes and lysosomes, which is elaborated in the following section (Diagram 1.14.) (Doray, Ghosh, Griffith, Geuze, & Kornfeld, 2002).

The transport of LMPs occurs through a M6PR independent pathway comprising either an indirect or direct route (Saftig & Klumperman, 2009). Most LMPs possess either a lysosome targeting di-leucine or tyrosine based sorting motif (Bonifacino & Traub, 2003; Braulke & Bonifacino, 2009), which permit these proteins to engage the AP1-clathrin and GGA- clathrin dependent direct route from TGN to endocytic pathway and eventually lysosomes (Höning, Griffith, Geuze, & Hunziker, 1996). Various studies have however demonstrated LMPs to be targeted to lysosomes in the absence of

AP1, providing evidence for a clathrin-coat independent route to lysosomes (Karlsson & Carlsson, 1998). The more elaborate path for LMPs to reach lysosomes is the indirect route, where LMPs are first transported to the PM via the constitutive secretory pathway and subsequently re-enter the cell by fluid-phase endocytosis (Saftig & Klumperman, 2009). This route to lysosomes starts at the PM and ends at the lysosomes. Between both of these locations lie trafficking intermediates and collectively, transport between these compartments involves a maturation process which includes, in temporal order of occurrence: early endosomes (EEs), and late endosomes (LEs) (Huotari & Helenius, 2011). These two stages differ in their functional and biological characteristics. The EE receives incoming endocytic vesicles from the PM that is then sorted into either cargo to be recycled back to the PM or material that requires further transportation along the endocytic pathway (van Meel & Klumperman, 2008). The main decision the EE has to make is whether the cargo it receives needs to be recycled or passed on to lysosomes (for degradation or functional purposes). This task is fulfilled by the transfer of recycling material to a distinct endocytic compartment, the recycling endosome (also known as tubular sorting endosomes, TSEs) and from there trafficked back by vesicular transport to the PM or TGN. Cargo destined for lysosomes (material to be degraded or lysosome resident proteins) remain in the EE for the next maturation process (van Meel & Klumperman, 2008). The next stop on route the lysosome is the LE where further sorting events take place. The progression from EE to LE is marked by the dynamic association of distinct small GTPases with LEs losing Rab5 and recruiting Rab7 (Poteryaev, Datta, Ackema, Zerial, & Spang, 2010; Rink,

Ghigo, Kalaidzidis, & Zerial, 2005). The ESCRT machinery regulates cargo sorting into intraluminal vesicles (ILV), these are cargo, which will be routed to lysosomes (Saksena, Sun, Chu, & Emr, 2007). LE intermediates also known as multivesicular bodies (MVBs) are vacuoles with numerous ILVs. LEs are characterized by elevated levels of proteins that are destined for lysosomes and substantial levels of hydrolases, whereas they contain only very low levels of recycling cargo (Saftig & Klumperman, 2009). In summary, LMPs are sorted in the TGN and can follow a direct or indirect transport pathway to the lysosomes (Diagram 1.14.).

LMP assembly and transport is tightly regulated, as proper lysosome function is highly dependent on these proteins. Mutation or absence of any LMPs in the lysosomal membrane can drastically interfere with lysosome function. Lysosome associated membrane proteins (LAMPs) are type 1 transmembrane proteins (single transmembrane span with luminal amino terminal and cytosolic carboxyl terminal orientation), which share high sequence homology. They are large proteins containing a heavily glycosylated luminal domain and short cytosolic tail (Saftig & Klumperman, 2009). LAMP1 expression at the cell surface is increased during platelet activation, on peripheral blood monocytes, cytotoxic T-cells and also in highly malignant tumour cells (Fukuda, 1991; Kannan et al., 1996). The underlying mechanisms for increased LAMP1 expression on the surface is not well understood, however expression of LAMPs on peripheral blood monocytes and cytotoxic T-cells contributes towards migration and targeting of leukocytes to the site of inflammation thereby playing a protective role (Kannan et al., 1996).

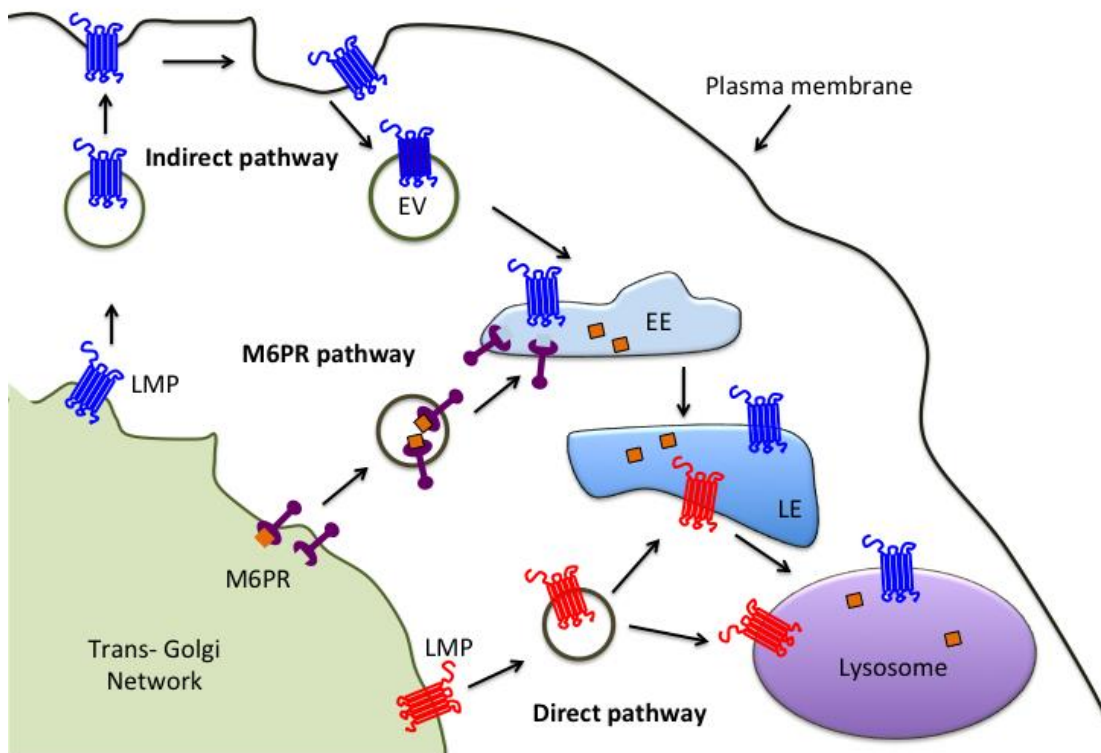


Diagram 1.14. The delivery of lysosomal membrane proteins and hydrolases to the lysosome. Newly synthesised membrane proteins at the trans- Golgi network (TGN) destined for lysosomes can be delivered by a constitutive ‘indirect’ pathway, where cargo first gets delivered from the TGN to the plasma membrane and from there is endocytosed and travels through the endocytic pathway (early endosome (EE) to late endosome (LE)) to ultimately reach the lysosome. The direct pathway employs inherent tyrosine or di-leucine based primary sequence motifs found in most LMPs, which target LMPs for sequestration into clathrin coated or non-clathrin coated vesicles, which fuse with LEs or lysosomes directly. Soluble lysosomal hydrolases are trafficked by the Mannose- 6 phosphate receptor (M6PR) pathway, which eventually delivers components to LEs and from there to lysosomes.

However up regulation of LAMP1 surface expression on tumour cells has been highly correlated with increased metastatic potentials (Agarwal et al., 2015). These findings collectively suggest a role for LAMP1 in cell adhesion and migration.

Furthermore studies in LAMP deficient cells display an altered lysosome distribution. Compared to their typical disperse and microtubule organisation centre localisation found in normal cells, LAMP deficient cells display a more peripheral positioning, suggesting that lysosome migration is delayed or disrupted upon LAMP depletion. LAMP1 deficient cells were also examined for lysosome motility upon pH changes, since this is an important contributor to lysosome positioning. Cytosolic pH contributes towards lysosome centrifugal and centripetal movement (Heuser, 1989). At acidic pH kinesin motor activity predominates and allows centrifugal movement, whereas under alkaline conditions dynein- centripetal localisation dominates. Results revealed that the dispersed lysosome localisation observed in LAMP1 deficient cells was independent of pH. One interpretation of these observations is that the short 11 aa C-terminal tail of the LAMP protein directly or indirectly interacts with the dynein-mediated centripetal migration system (K. K. Huynh et al., 2007).

1.4.2. Lysosome functions

The most widely studied function of lysosomes is the catabolic turnover of cellular debris, proteins and damaged organelles (Luzio et al., 2007). More recently, additional important cellular functions of lysosomes have emerged that have expanded the influence of these organelles into many other areas

of cell biology. Lysosomes have now been shown to have functions in PM repair, cellular homeostasis, energy metabolism and immune responses, therefore their functions can be divided into three broad categories: 1) Catabolism, 2) Signalling and 3) Secretion (Settembre et al., 2013). Interestingly, aberrant lysosome activity is often observed in transformed cells and is almost always pro-oncogenic and therefore lysosomes have, in recent years, become attractive potential future therapeutic anti-cancer targets (Gyparaki & Papavassiliou, 2014; Kirkegaard & Jäättelä, 2009).

1.4.2.1. The endocytic pathway and lysosomal transport

The most widely studied function of lysosomes is the proteolysis of extracellular material including ligands and PM proteins. There are two primary routes for transporting material for degradation to the lysosomes. The endocytic pathway collects extracellular waste material for delivery to lysosomes, whereas intracellular waste is delivered to lysosomes by the process of autophagy (Settembre et al., 2013).

The pathway from internalisation of extracellular material to lysosomal delivery is known as the endo-lysosomal pathway (Grant & Donaldson, 2009). The endo-lysosomal pathway is balanced with the endosomal recycling pathway, which mediates return of selected materials to earlier compartments along the pathway and the PM.

The uptake of extracellular material or PM receptors is known as endocytosis and can be divided into clathrin- dependent endocytosis (CDE) or clathrin-independent endocytosis (CIE) (Le Roy & Wrana, 2005). CDE is a very specialised targeted mode of endocytosis as it works via highly specific

adaptor proteins, which recognise the cytoplasmic domain of the target PM protein. On adaptor binding, the specific target protein is internalised within a clathrin coated vesicle for transport within the cell. Examples of this mode of endocytosis are internalisation of transferrin receptor and low-density lipoprotein (Le Roy & Wrana, 2005). Various accessory proteins and dynamin GTPases are required for vesicle scission. Once the clathrin coat has disassembled, vesicles contents can be trafficked to lysosomes for degradation via entry into the endocytic pathway described previously.

The alternative internalisation process is CIE, which depends on lipid modification, BAR (Bin/Amphiphysin/Rus) proteins and insertion of proteins into the PM allowing invagination and providing a platform for vesicle formation. Additionally actin, actin- associated proteins, sorting nexin family and sorting nexin 9 are important factors in CIE vesicle formation (Mayor & Pagano, 2007). There are several forms of CIE depending on the proteins involved in vesicle biogenesis such as RhoA/Cdc42, Arf6, and caveolin (Sandvig, Pust, Skotland, & van Deurs, 2011). CIE usually operates in membrane and fluid internalisation. Whatever the CIE form is, the internalised components are packaged in vesicles and enter the endocytic pathway for eventual degradation by lysosomes.

The trafficking pathway from EE to LE has been studied extensively (Huotari & Helenius, 2011) however the step from LE to lysosome is not fully elucidated as it is a dynamic process that has complicated its study. The hallmark of endosome to lysosome progression is the significant luminal acidification (Settembre et al., 2013).

Dynamic fusion and fission events occur constantly between LE and lysosomes with partial content integration and temporary hybrid organelle biogenesis, therefore strict delineation between LE and lysosomes, and studying these organelles separately, is extremely challenging. There exist three hypotheses concerning how content trafficking between LE and lysosomes could occur (Diagram 1.15.). The first of these is based on vesicular transport; content is transported via vesicles between the two organelles. The second is termed “kiss and run”. In this mode LEs and lysosomes undergo continuous but transient fusion and fission events whereby content can be exchanged. The third option is the direct and complete fusion between the two organelles with subsequent recovery of lysosomes for re-use (Luzio et al., 2007).

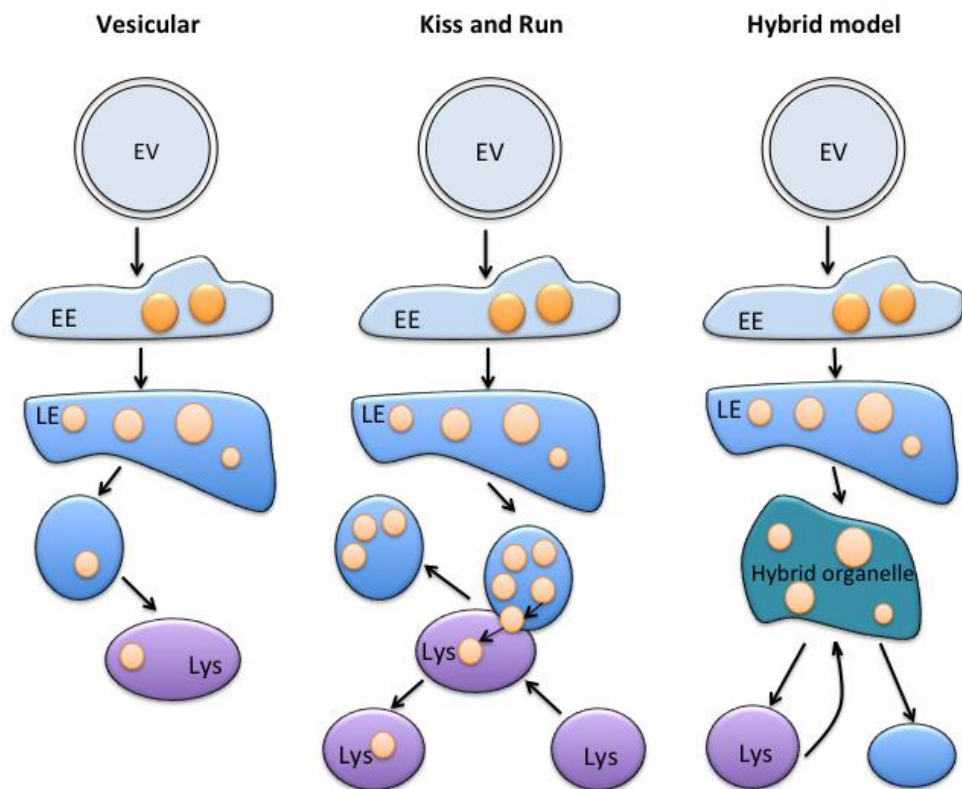


Diagram 1.15. The endocytic pathway and possible mechanisms for delivery of material to lysosomes. Internalised cargo from the plasma membrane is initially sorted from endocytic vesicles (EV) into early endosomes (EE), then late endosomes (LE) and finally to lysosomes (Lys) for degradation. There are three different models for LE to Lys trafficking. From left to right: The vesicular model proposes cargo containing vesicles bud from LEs and are delivered to Lys' via transport vesicles; the kiss and run model suggests a temporary part fusion between LEs and Lys', whereby cargo is transferred to the Lys lumen and in the hybrid model LEs and Lys' completely fuse to form a hybrid organelle.

1.4.2.2. Lysosomes and Autophagy

Autophagy is an evolutionarily conserved homeostatic mechanism, which delivers cytoplasmic material and organelles to lysosomes for bulk degradation. Autophagy can be up-regulated during periods of cellular stress including nutrient starvation and oxidative stress but under normal conditions

also plays a role in general cellular housekeeping (removal and recycling of aged or malfunctioning cellular components), apoptosis and immune responses.

There are three distinguishable types of autophagy: Macroautophagy/ Autophagy; Microautophagy and Chaperone- mediated autophagy (CMA) (Diagram 1.16.) (Eskelinen & Saftig, 2009; Glick, Barth, & Macleod, 2010). Macroautophagy (MA) is operational at low levels in most cells for homeostatic purposes, however MA is strongly induced by stress signals. The purpose of MA is to deliver damaged or redundant cytoplasmic cargo to lysosomes for degradation. The very first step in MA is autophagosome formation, whereby the isolation membrane/ phagophore engulfs target cargo or a portion of the cytoplasm to form a double membrane enclosing it. After initial phagophore formation a stepwise maturation process occurs. Autophagosomes can fuse with endosomes or lysosomes directly to generate an amphisome or autolysosome respectively and eventually the contents of these hybrid organelles are degraded by lysosomal hydrolases. The degradation by-products are usually recycled back to the cytosol for re-use (Eskelinen & Saftig, 2009). For example, during nutrient starvation, MA can catabolise cellular material to provide the building blocks required to maintain critical cellular functions. The molecular mechanism of final lysosome fusion still remains to be understood in detail. An early requirement for the small GTPase Rab7 and presenilin proteins has been established (Gutierrez, Munafó, Berón, & Colombo, 2004). At later stages cytoskeleton and the lysosomal membrane proteins LAMP1 and LAMP2 along with lysosomal hydrolases Cathepsin B and D are also essential for this process

(Glick et al., 2010). The molecular mechanism of MA is most widely studied and known to be tightly regulated by various autophagy specific *atg* gene products (Glick et al., 2010).

Microautophagy is a non-selective process whereby the lysosomal membrane itself directly sequesters a small portion of the cytoplasm or cargo for degradation. The main purpose for this is likely organelle size maintenance and membrane homeostasis (W.-W. Li, Li, & Bao, 2012).

CMA is a highly selective and direct delivery route for cargos to the lysosomal lumen. Only proteins possessing a consensus peptide sequence (KFERQ) are recognised by the 70kDa cytosolic chaperone heat shock cognate protein (hsc70), which allows interaction with the lysosome membrane and finally transfer of bound cargo to the lysosomal lumen by chaperones located on the luminal side (W. Li, Yang, & Mao, 2011). The CMA substrate recognition motif is KFERQ, which has been shown to bind to hsc70. Once in complex with hsc70, substrate protein is targeted to the lysosomal surface by interaction with the cytosolic tail of LAMP2. In order to cross the lysosomal membrane the substrate needs to be unfolded and internalised into the lysosomal lumen which is facilitated by LAMP2 multimerisation, hsc70 and co-chaperones (Cuervo & Wong, 2014). This process is reminiscent of the retro translocation of ERAD substrates across the ER membrane for 26S proteasomal degradation (Vembar & Brodsky, 2008). CMA is essential for clearance of aged, dysfunctional or incorrectly synthesized/folded proteins which could be potentially cytotoxic and hence functions as part of the cellular quality control surveillance system (Glick et al., 2010).

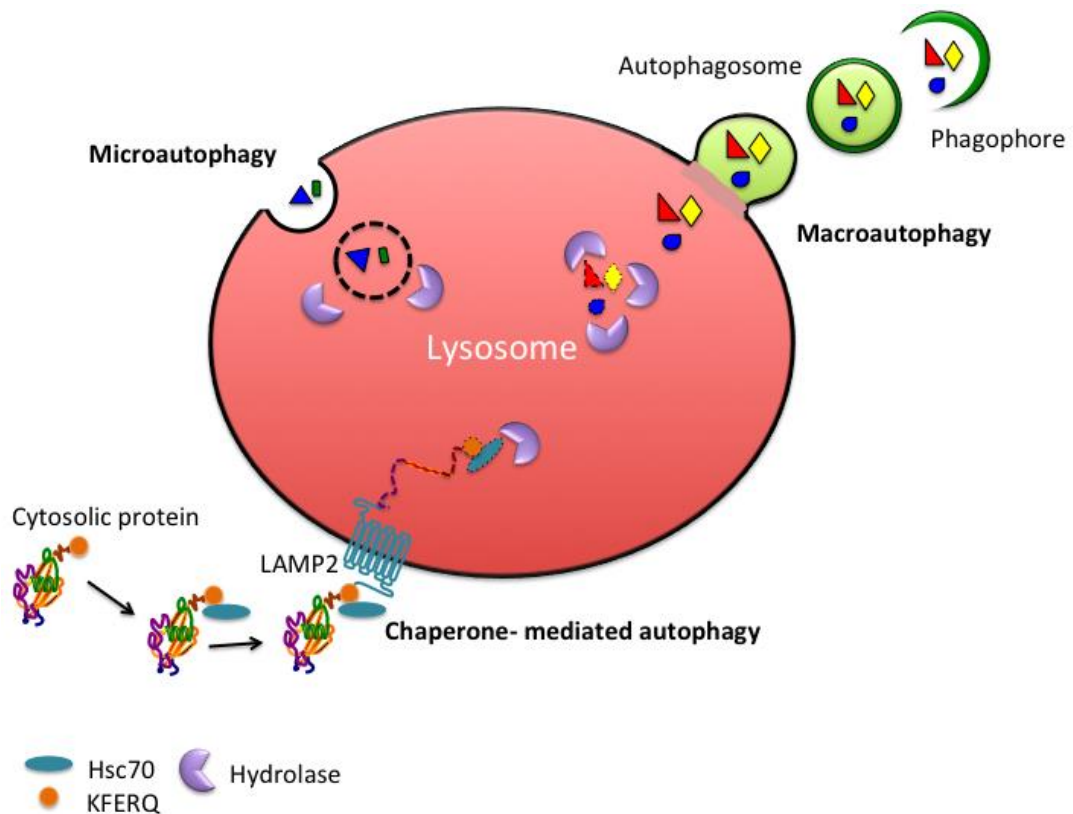


Diagram 1.16. Classes of autophagy and how they deliver material to lysosomes. Cytoplasmic material for degradation can be delivered to lysosomes via three different routes. The simplest route is the direct engulfment by the lysosomal membrane of cytoplasmic material known as microautophagy. In Macroautophagy a phagophore membrane is first formed which begins to engulf an entire volume of cytoplasm, this structure eventually seals completely to form a fully membrane bounded autophagosome. The autophagosome eventually fuses with the lysosome resulting in content degradation. Proteins containing the recognition sequence KFERQ are targeted by the chaperone-mediated pathway to the lysosomes where LAMP2 membrane protein on lysosomes facilitates transfer to the lysosome lumen for eventual degradation.

CMA mediated degradation takes place stepwise and encompasses substrate recognition and lysosomal targeting followed by substrate binding and unfolding to allow translocation into the lysosomal lumen and finally, degradation. Interestingly, it has recently been reported that a defect in

autophagy under conditions of nutrient stress is linked to mitosis defects in dividing yeast cells. The essential amino acids provided by this process under such circumstances permits normal mitosis and in autophagy deficient strains mitotic failure and an increase in the frequency of aneuploidy cells was observed (Matsui, Kamada, & Matsuura, 2013).

1.4.2.3. Lysosome exocytosis

Lysosomal exocytosis has been implicated in immune responses, bone resorption, cell signalling and plasma membrane repair (Andrews, 2000; 2005). Lysosome fusion with the PM can be tracked by the appearance of the luminal domain of LAMP1 on the extracellular face of the PM (A. Reddy, Caler, & Andrews, 2001). This mechanism is Ca^{2+} dependent and requires the ubiquitously expressed Ca^{2+} sensing LMP synaptotagmin 7 (SYT7, (Idone et al., 2008). SYT7 interacts with SNARE proteins to mediate lysosome and PM fusion. VAMP7 is the vesicle- SNARE (v-SNARE) that forms a complex with syntaxin4 and SNAP23 that are specific PM target-SNAREs (t-SNAREs). This SNARE complex interacts with SYT7 in a Ca^{2+} dependent manner to mediate Ca^{2+} dependent lysosome exocytosis (S. K. Rao, Huynh, Proux-Gillardeaux, Galli, & Andrews, 2004). LAMPs, VAMP7 and SYT7 have also been shown to be required for Ca^{2+} dependent phagosome-lysosome fusion (K. K. Huynh et al., 2007; Saftig & Klumperman, 2009).

PM injuries can frequently occur in cells operating under mechanical stress or upon damage induced by chemical toxins and certain pathogens (Rodríguez, Webster, Ortego, & Andrews, 1997; Roy et al., 2004). Since

Ca^{2+} from the extracellular medium can enter the cell upon membrane integrity loss, rapid closure of membrane perforations is an essential damage limitation mechanism. Lysosome exocytosis is triggered under such circumstances and has been shown to efficiently reseal the PM and restore its functional integrity. Dynamic insertion of cellular membrane at cytokinesis is, as discussed previously, essential for normal separation of daughter cells and lysosomes might play a role in this function.

1.4.3. Lysosome function and disease

Aberrant lysosomal activity underpins a number of human diseases of which the lysosomal storage diseases (LSDs) are the most common ones. The incidence of LSDs is estimated at around 1:5000 live births (Mehta et al., 2006) and the common feature of all LSDs are mutations affecting specific lysosomal proteins and their associated activities (Saftig & Klumperman, 2009). The malfunctioning of these proteins triggers the accumulation of undegraded substrates in various compartments of the endocytic-autophagic-lysosomal system (Platt, Boland, & van der Spoel, 2012).

The severity of LSDs depends on the affected protein, the biochemistry of the non-degraded substrate material and the cell type where the storage defect occurs. The clinical manifestation of LSDs is usually a neurodegenerative disease at childhood, however storage diseases in bone or cartilage can appear normal at birth with later diagnosis and manifestations (Settembre et al., 2008). LSDs are in general quite serious and severe diseases causing premature death.

Lysosomes are also receiving increasing interest in the field of oncology/cancer research because of their newly discovered links to tumour progression. Lysosomes and their normal functions are frequently altered in malignant transformed cells (Gyparaki & Papavassiliou, 2014). Observed changes include enhanced lysosome biogenesis, hydrolytic activity, lysosomal content secretion and modifications to the composition of the lysosome membrane. Lysosomes also furnish certain cancer cell types with resistance to chemotherapeutic agents. Lysosomes are capable of accumulating anti- cancer drugs in their acidic lumen thereby attenuating their efficacy (Groth-Pedersen & Jäättelä, 2013; Kallunki, Olsen, & Jäättelä, 2013). Lysosomal hydrolytic enzymes and membrane proteins have been found in many studies to be involved in tumour progression and/or inhibition (Appelqvist et al., 2013; Kirkegaard & Jäättelä, 2009). Pro-oncogenic properties have been directly linked to increased cathepsin activity. Overexpression of cathepsin B and S for instance has been shown to promote cancer progression whereas depletion elicited tumour growth inhibition, indicating direct involvement of these hydrolases in cellular transformation and tumorigenesis (Bengsch et al., 2014; Gondi & Rao, 2013; Small et al., 2013).

Additionally lysosomes are involved in programmed cell death by releasing certain cathepsins into the extracellular space, which initiates apoptotic cell death under pathological conditions such as degenerative and infectious diseases (Boya & Kroemer, 2008; Guicciardi, Leist, & Gores, 2004). Interestingly lysosome initiated cell death is prevented in numerous cancer cells upon heat shock protein 70 (hsp70) overexpression, which is commonly

upregulated in many tumours (Gyrd-Hansen, Nylandsted, & Jäättelä, 2004; Nylandsted et al., 2004). Cancer cells rely on hsp70 survival actions and therefore hinder cancer cell death, allowing uncontrollable cell division (Kirkegaard & Jäättelä, 2009).

1.5. Summary

Ca^{2+} signalling influences almost all aspects of mammalian cell biology and its versatility stems from the expression of large numbers of Ca^{2+} sensing proteins and a complex machinery capable of generating and terminating exquisitely tailored signalling patterns. The CaM related family of small EF-hand containing Ca^{2+} sensors are the largest family involved in transduction of Ca^{2+} signals and controlling specific aspects of cellular physiology. CaBPs are relatively recently discovered CaM related proteins and much remains to be learnt regarding their cellular functions. This thesis focuses on CaBP7, a vertebrate specific protein that has distinctly evolved (along with the highly related CaBP8) from the other CaBPs and the only small Ca^{2+} binding protein, other than CaM, to have a documented function during mammalian cell cytokinesis. Mitosis and cytokinesis are responsible for allowing eukaryotic cells to divide with high fidelity, a process of fundamental importance to normal development, tissue regeneration and aging of complex multicellular organisms. Misregulation of cytokinesis can induce a state of aneuploidy, which is associated with enhanced tumorigenesis and potentially catastrophic consequences for the organism. Understanding the fundamental molecular mechanisms governing this process are therefore of

great importance in modern cell biology research. With the knowledge that CaBP7 can influence cytokinesis the work in this thesis has been directed at trying to understand the molecular basis for this activity. CaBP7 is an inhibitor of PI4KIII β and this enzyme has documented functions in controlling cargo transport from the Golgi complex. Interestingly, this kinase has also been implicated in the regulation of mitosis in model organisms. In addition to the Golgi complex, PI4KIII β has also recently been localised to lysosomes, organelles, which are also able to influence cell division. Taking these observations into consideration the work in this thesis has been directed at trying to understand the potential interplay between regulation of PI4KIII β activity by CaBP7, how this could occur on lysosomes and how this functionally impacts on normal cytokinesis. Specifically, the studies reported in this work have addressed:

1. The detailed subcellular localisation of CaBP7
2. If CaBP7 is able to regulate lysosomal PI4KIII β
3. The distribution of CaBP7, PI4KIII β and lysosomes during mitosis
4. How cellular depletion of CaBP7 impacts on PI4KIII β activity and lysosome trafficking during mitosis in mammalian cells
5. The functional requirement of lysosomes during cell division.

Collectively these analyses have provided intriguing links between CaBP7 regulation of PI4KIII β on lysosomes during mitosis and cytokinesis in mammalian cells and indicate that correct localisation of lysosomes at cytokinesis is essential for normal completion of mitotic cell division. This work provides new insights into regulation of mitosis and has opened further avenues of investigation particularly into a potentially novel functional role of lysosomes during this fundamental process.

Chapter 2

Materials and Methods

Materials

Table 2.1. Reagents and suppliers

All reagents were of laboratory analytical grade and obtained from Sigma (Poole, UK) unless otherwise stated. Other suppliers are listed below.

Supplier	Address
Abcam	Cambridge, UK
Bioline	Sheffield, UK
BioRad	California, USA
Cayman	Utah, USA
Echelon	Michigan, USA
Invitrogen/Life Technologies	Paisley, UK
Leica	Heidelberg, Germany
MatTek	Massachusetts, USA
Millipore	Livingston, UK
NEB (New England Biolabs)	Hertfordshire, UK
Origene	Cambridge, UK
Promega	Southampton, UK
PromoCell	Heidelberg, Germany
Qiagen	Manchester, UK
Roche	Hertfordshire, UK
Santa Cruz	Texas, USA
Thermo Scientific	Winsford, UK
Tocris	Bristol, UK
Zeiss	Jena, Germany

Table 2.2. Recipes of buffers and media

Buffer	Recipe
ECL Reagents (Enhanced Chemi Luminescence Reagents)	<p><i>ECL solution A:</i> 100 mM Tris HCL (pH 8.5) 2.5 mM Luminol 0.4 mM p-coumaric acid</p> <p><i>ECL Solution B</i> 100 mM Tris HCL (pH 8.5) 0.02% (v/v) H₂O₂</p>
IF Blocking Buffer (Immunofluorescence blocking buffer)	PBS plus 5% (w/v) Bovine Serum Albumin- lyophilized powder (BSA)
LB Agar (Luria broth Agar)	1.5% (w/v) bacto-agar 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 8.56 mM NaCl
LB PBS-T (Lipid blot PBS-Tween)	PBS plus 0.1% (v/v) Tween-20
LB Media (Luria Broth Media)	1% (w/v) tryptone 0.5% (w/v) yeast extract 8.56 mM NaCl

<p>PBS (Phosphate buffered saline)</p>	<p>137 mM NaCl 2.7 mM KCl, 10 mM Na₂PO₄ 2 mM NaH₂PO₄ pH 7.4</p>
<p>PBS-T (PBS- Tween)</p>	<p>PBS with 0.05% (v/v) Tween-20</p>
<p>4x SDS buffer (4x Sodium Dodecyl sulphate buffer)</p>	<p>4% (w/v) SDS 2 mM EDTA 10% (w/v) Sucrose 125 mM HEPES 1% (v/v) β –Mercaptoethanol 10% (v/v) Glycerol pH 6.8</p>
<p>SOC Media (Super Optimal broth with Catabolite repression media)</p>	<p>2% (w/v) tryptone, 0.5% (w/v) yeast extract 8.56 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂ 20 mM glucose</p>
<p>TAE (Tris base, acetic acid and EDTA)</p>	<p>40mM Tris Base pH 8.0 20 mM acetic acid 1mM EDTA</p>

<p>TBS (Tris buffered saline)</p>	<p>50 mM Tris HCl 150mM NaCl pH 7.4</p>
<p>WB Blocking solution (Western blot blocking solution)</p>	<p>PBS plus 3% (w/v) skimmed milk powder</p>

Methods

2.2. Molecular Biology

2.2.1. Polymerase chain reaction (PCR)

In order to sub clone DNA encoding specific sequences from existing plasmids into new plasmids PCR reactions were performed with oligonucleotide primer pairs containing specific restriction endonuclease sites. Each PCR mix was prepared in a total of 100 µl and comprised: 20 µl 5x PCR reaction/ Phusion ® buffer, 5% (v/v) DMSO, 0.5 µM of both forward and reverse primers (for CaBP7 QPCR: sense 5' atataagcttatgccgttcacccgg 3', anti-sense 5' atatgaattcctacttcatgccactgcgc 3'), 0.2 mM deoxyribonucleoside triphosphates (dNTPs), 0.5 µg DNA template and 1U Phusion ® polymerase (NEB) and distilled water was added to obtain a total of 100 µl. The PCR cycling parameters were as follows:

Step 1 Denature	98°C 1 minute (1 cycle)	
Step 2 Denature	98°C 10 seconds	} 30-35 cycles
Annealing*	T_m -5-10°C 10 seconds	
Extension	72°C 15 seconds/kilo base	
Step 3 Run off	72°C 10 minutes (1 cycle)	
Hold	4°C	

*The annealing temperature was determined depending on the primer pairs lowest melting temperature (T_m).

2.2.2. Purification of PCR products

PCR products were purified with the QIAquick® PCR purification kit (Qiagen) or Wizard® SV Gel and PCR clean- up system (Promega) following the manufacturer's protocol. In short: An equal volume of membrane binding solution was added to the PCR product. This mixture was then transferred to a silica based spin minicolumn and incubated for 1 minute at room temperature (RT). The silica membrane with bound DNA was washed twice to remove primers, dNTPs and protein. Pure DNA was finally eluted from the minicolumn by adding nuclease-free water to the column and centrifugation at 13,000 rpm into a sterile collection tube.

2.2.3. Restriction digests

PCR products (inserts) and target plasmids with complimentary restriction digest overhangs were generated as follows: 1 µg of both plasmid and insert were prepared for restriction digest in a total reaction volume of 50 µl which

comprised 5 µl of 10x appropriate restriction enzyme Buffer 1U of each restriction enzymes (NEB) 5 µl of 10x BSA and distilled water to 50 µl. Restriction digests were incubated for 1 hour at 37°C. Endonuclease treated samples were subsequently resolved by horizontal agarose gel electrophoresis and staining with SYBR[®] Safe DNA stain (Life Technologies).

2.2.4. Agarose gel electrophoresis and gel extraction of nucleic acid

Agarose gels were prepared by dissolving low melting temperature high purity agarose (Bioline) at a concentration of 1% (w/v) in 1x TAE buffer by brief boiling in a microwave oven. Once cooled to ~40-50°C 0.5 µl/ml SYBR[®] Safe (Invitrogen) was added to the solution before pouring into a prepared gel cast. 5x loading dye (Bioline) was added to DNA samples to a concentration of 1x and samples loaded onto the gel. 5µl of Hyperladder[™] I DNA molecular weight marker (Bioline) was also loaded onto the gel. Samples were resolved at 80V for 30-45 minutes after which DNA was visualized under UV illumination and appropriate DNA fragments excised from the gel using a sterile scalpel. QIAquick[®] gel extraction kit (Qiagen) or Wizard[®] SV Gel and PCR clean- up systems (Promega) were employed to extract DNA from gel slices. In brief: excised agarose bands were dissolved in membrane binding buffer by heating at 55°C for 5 minutes. Dissolved DNA was bound to a silica spin minicolumn and washed twice to remove excess salts. Subsequently pure DNA was eluted from the column in nuclease-free water by centrifugation at 13,000 rpm for 1 minute into a sterile collection tube.

2.2.5. Ligation of DNA fragments

Ligation reactions allow the covalent linking of two linearized DNA fragments to form a circular species. The two digested and gel extracted linearized DNA fragments to be ligated were prepared in a 20 µl ligation mix that included: 100ng linearized plasmid DNA; insert cDNA (at 1:2, 1:3, 1:6 molar ratios (vector: insert); 2 µl 10x T4 DNA ligase buffer (NEB); 400U T4 DNA ligase (NEB) and distilled water to a final volume of 20 µl. These reaction mixes were incubated at RT for 20 minutes and 3 µl transformed into 50 µl competent *E.coli* bacteria (DH5α cells).

2.2.6. Transformation of chemically competent *E.coli*

For amplification of plasmid DNA either 0.5 µl of purified plasmid or 3 µl of ligation mixes were transformed into 50 µl of chemically competent DH5α *E.coli* cells. Cells were then incubated on ice for 30 minutes and subsequently heat shocked for 45 seconds at 42°C. Samples were transferred to ice for 2 minutes and then 450 µl of RT SOC media was added and cells incubated at 37°C for 1 hour with continuous shaking at 250 rpm. Transformed bacteria were then plated under sterile conditions onto LB agar containing an appropriate antibiotic for selection of transformed cells (Table 2.3.). Plates were inverted and placed in a 37°C incubator overnight to facilitate bacterial growth. 24 hours post transformation colonies were picked using sterile pipette tips and transferred to liquid LB cultures for larger scale preparation of DNA.

2.2.7. Screening for successful ligation reactions

Single colonies from a ligation plate were picked with a sterile pipette tip and used to inoculate 5 ml of LB media containing the appropriate antibiotics (Table 2.3.). Cultures were grown overnight at 37°C/225 rpm. The following day cells from bacterial cultures were collected by centrifugation (2500 x *g* for 5 min) and plasmid DNA extracted using a spin column mini prep kit (Promega) according to the manufacturer's protocol. Briefly, cells were lysed with an alkaline solution containing sodium dodecyl sulphate (SDS) and immediately neutralized by acetate addition to precipitate genomic DNA and SDS. The suspension was subsequently centrifuged (12,000 x *g* for 5 minutes) to pellet precipitated material and the supernatant transferred to a silica gel based spin column to capture the DNA. Bound DNA was washed to remove impurities and finally eluted into 30-50 µl Tris based buffer.

Purified DNA was verified by restriction endonuclease digestion followed by agarose gel electrophoresis and DNA sequencing in both the forward and reverse directions.

Following confirmation of correct ligation, plasmids were re-transformed into DH5α *E. coli* for large scale plasmid purification. Briefly, single colonies were picked and used to inoculate 50-150 ml LB media supplemented with the appropriate antibiotic (Table 2.3.). Cultures were grown overnight at 37°C/225 rpm. Cells were harvested by centrifugation (3800 rpm/10 min/4°C) and plasmid purified with a PureLink HiPure Plasmid Midi/Maxi Kit (Invitrogen) following the manufacturer's protocol. Purified DNA

concentration was determined with a NanoDrop Lite Spectrophotometer (Thermo Scientific).

Table 2.3.: Antibiotics used for plasmid selection

Antibiotics	Stock concentration	Working concentration
Kanamycin	30 mg/ml	30 µg/ml
Ampicillin	100 mg/ml	100 µg/ml

2.2.8. RNA isolation from HeLa cells

HeLa cells were grown on 10 cm cell culture dishes to confluency. Cells were washed twice with ice cold PBS before addition of 1 ml TRIzol reagent (Invitrogen). Wells were scraped extensively to ensure removal of all cells and samples incubated for 5 minutes at RT. 200 µl Chloroform was added and samples vigorously shaken by hand for 15 seconds followed by incubation at RT for a further 3 minutes. Samples were centrifuged at 12,000 x *g* for 15 seconds/4°C, resulting in separation into 3 distinct phases: a lower red phenol/chloroform phase, an interphase (precipitated material) and a colourless upper aqueous phase. The aqueous phase was collected into a clean tube and 500 µl isopropanol added to precipitate RNA. The sample was then centrifuged at 12,000 x *g* for 10 minutes/4°C. The resulting pellet was washed with 1ml 75% (v/v) ethanol and subsequently dried at RT for 10 minutes. The dried pellet was finally re-suspended in 35µl RNA/DNAase free water and incubated at 55°C for 10-15 minutes.

2.2.9. Reverse Transcription/ cDNA preparation

Complementary DNA (cDNA) was prepared using Bioscript™ Reverse Transcriptase (Bioline). Priming mix was prepared as follows: 3µg RNA, 1µl Random Hexamers, 1 µl 10 mM dNTPs and distilled water in a total of 10µl. The mixture was incubated at 70°C for 5 minutes followed by 1 minute on ice. The reaction mix was prepared in a total of 20 µl: 5x RT buffer, 1 µl Ribosafe RNase Inhibitor, 200U BioScript Reverse Transcriptase to total volume of 20µl. 10 µl of the reaction mix was added to 10 µl priming mix and transcription reactions gently mixed. Samples were incubated successively at 25°C for 10 minutes, 42°C for 30 minutes and finally 85°C for 5 minutes to terminate the reaction. The resulting single stranded cDNA was diluted 2 fold.

2.2.10. DNA Sequencing

All new expression plasmids were verified by automated dideoxy sequencing in both directions by The Sequencing Service, University of Dundee, UK.

2.3. Cell culture and transfections

2.3.1 HeLa cells maintenance

HeLa cells were maintained in DMEM media supplemented with 1% (v/v) Penicillin- Streptomycin, 1% (v/v) Non-essential amino acids and 10% (v/v)

Foetal bovine serum. Cells were always maintained at 37°C in a humidified environment of 5% CO₂/95% air.

2.3.2. Transfections of HeLa cells

Cells for transfections were plated onto either 6-well plates or 13mm glass coverslips placed into 24-well plates 24 hours prior to transfection at a density of approximately 5×10^5 cells/ml. For each µg of plasmid DNA to be transfected 3µl of Promofectin (PromoCell) transfection reagent was used according to the manufacturer's protocol. 1 µg of each plasmid was transfected into cells grown on 13 mm coverslips whereas 3 µg of each plasmid was transfected into cells plated onto 6-well trays (35 mm wells). Transfection mixtures (Plasmid plus transfection reagent diluted in RPMI 1640 media (100 µl/ 1 µg DNA mix) were vortexed for 10 seconds and incubated at RT for 30 minutes, before being added drop-wise to cells. Transfected cells were incubated for 24 hours at 37°C unless otherwise stated before processing for immunofluorescence, western blotting, lipid extraction or live- cell imaging.

2.4. SDS PAGE and Western blotting

Cells plated onto 6-well plates were transfected at 60-70% confluency and 24 hours post-transfection solubilized and lysed into 100µl 4x SDS buffer. Samples were briefly sonicated to reduce viscosity (3x 5 sec pulses at 12 % power output) and then boiled for 5 minutes. Proteins were resolved by SDS

PAGE on 4-12% Tris-glycine gradient Novex gels (Invitrogen). 5 µl of Geneflow pre-stained protein molecular weight marker was loaded along with boiled cell lysate samples. Proteins were resolved at 180V for approximately 45-60 minutes prior to transfer onto nitrocellulose membrane using the X-Cell II™ Blot Module (Life Technologies) at 30V for 1 hour. The nitrocellulose membrane was stained with Ponceau-S solution (Sigma) to confirm successful protein transfer and to permit trimming of excess membrane material. Ponceau-S was removed by 2x 10 min washes with PBS and the membrane incubated with WB blocking solution for 1 hour at RT. Primary antibodies at the appropriate dilution (Table 2.4.) were prepared in WB blocking solution and applied to membranes for 1 hour at RT or overnight at 4°C with constant agitation. Following 3x washes with PBS-T and PBS, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody in WB blocking solution for 1 hour at RT (Table 2.4.). Membranes were washed 3x with PBS-T and once with PBS and subsequently incubated with equal volumes of ECL reagents. Blots were developed and imaged on a ChemiDoc XRS system (BioRad).

Table 2.4.: Antibodies used for western blotting in this thesis

Antibody	Description	Supplier	Dilution
Anti-CaBP7	Rabbit polyclonal	Santa Cruz	1:200
Anti-RFP	Rabbit polyclonal	Prof Ian Prior	1:500
Anti-GAPDH	Mouse monoclonal	Sigma	1:1000
Anti- GFP	Mouse monoclonal	Roche	1:500
Anti- Calmodulin	Rabbit polyclonal	Abcam	1:1000

Anti- Mouse IgG HRP	Goat polyclonal	Sigma	1:500
Anti- Rabbit IgG HRP	Goat polyclonal	Sigma	1:500

2.5. Lipid Extraction and Dot blot

HeLa cells were plated onto 10 cm dishes, allowed to adhere overnight, and transfected (5µg mCherry-N1 or 5 µg mCherry-CaBP7) the following day. 24h post-transfection (density of $\sim 2.2 \times 10^6$ cells/dish) cells were trypsinised and collected into sterile 15 ml falcon tubes. Cells were homogenized with 1ml of Chloroform: Methanol (2:1 v/v) mixture. After dispersion, the mixture was agitated at RT on an orbital shaker for 15 minutes. The homogenate was centrifuged at $2400 \times g$ for 3 minutes to recover the liquid phase. The solvent was then washed with 200µl 0.9% (w/v) NaCl solution. Upon vortexing the mixture was centrifuged at $380 \times g$ to elicit phase separation. The upper aqueous phase was discarded and the interface surface gently rinsed twice with methanol: water (1:1 v/v) without disturbing the mixture. Subsequently, the lower organic phase was centrifuged for 45 minutes at 43°C degrees (medium drying rate) under vacuum to allow chloroform evaporation (SpeedVac SC210A). Lipid pellets were re-suspended in 50 µl Chloroform: Methanol (2:1 v/v). This sample was diluted in half twice using Chloroform: Methanol (2:1 v/v) to generate three lipid samples. 1 µl of each sample was spotted onto the unused central area of a PIP-Strip™ membrane (Echelon) and solvent allowed to completely evaporate. Once dried, the blot was incubated with LB blocking solution overnight at 4°C. The following day the membrane was incubated with 0.5µg/ml PI4P Grip™ (Echelon) protein in LB

blocking solution for 1 hour at RT with gentle agitation. After 3 washes with LB PBS-T, the blot was incubated with anti-GST (Rabbit polyclonal -Sigma) diluted 1:500 in LB blocking solution for 1 hour at RT with gentle agitation. Upon 3 washes with PBS-T, the blot was then incubated with anti-rabbit IgG HRP diluted 1:500 in LB blocking solution for 1 hour at RT with gentle agitation. The membrane was washed as previously described and visualized with the ECL detection method. PIP Strips [™] are 2 x 6 cm hydrophobic membranes that have been spotted with 100 pmol of all eight phosphoinositides and seven other biological important lipids. The layout of the PIP-Strip [™] is shown below:

<u>Lipid Membrane Strips</u>			
Triglyceride	○	○	Phosphatidylinositol (PI)
Diacylglycerol (DAG)	○	○	PtdIns(4)P
Phosphatidic Acid (PA)	○	○	PtdIns(4,5)P ₂
Phosphatidylserine (PS)	○	○	PtdIns(3,4,5)P ₃
Phosphatidylethanolamine (PE)	○	○	Cholesterol
Phosphatidylcholine (PC)	○	○	Sphingomyelin
Phosphatidylglycerol (PG)	○	○	3-sulfogalactosylceramide (Sulfatide)
Cardiolipin	○	●	Blue Blank

Reproduced from Echelon website (www.echelon-inc.com) Lipid Membrane Strips [™] catalogue P-6002 (Echelon).

2.6. Plasmid constructs

Table 2.5.: Plasmids and fluorescent proteins used in this thesis

Plasmid	Origin	Resistance
mCherry-N1	Dr Lee Haynes	Kanamycin
mCherry-CaBP7	Dr Lee Haynes	Kanamycin
mCherry-CaBP7	Dr Hannah McCue	Kanamycin

EF1		
mCherry-CaBP7	Dr Hannah McCue	Kanamycin
EF2		
mCherry- CaBP7	Dr Hannah McCue	Kanamycin
EF1 + EF2		
mCherry-CaBP7 shRNAi resistant	Dr Hannah McCue	Kanamycin
NCS1- mCherry	(Handley, Lian, Haynes, & Burgoyne, 2010)	Kanamycin
CaBP4- mCherry	Dr Lee Haynes	Kanamycin
LAMP1-mCherry	Dr Lee Haynes	Kanamycin
H2B-mCherry	Addgene	Ampicillin
mCherry-Arl8b	Dr Lee Haynes	Kanamycin
mCherry-Arl8b Q75L	Dr Lee Haynes	Kanamycin
EYFP-N1	Clontech	Kanamycin
YFP-CaBP7	Dr Lee Haynes	Kanamycin
NCS1-YFP	Dr Lee Haynes	Kanamycin
ARF1- YFP	Dr Lee Haynes	Kanamycin
LAMP1- YFP	Dr Lee Haynes	Kanamycin
LC3-YFP	Dr Lee Haynes	Kanamycin
Rab5a-YFP	Dr Lee Haynes	Kanamycin
HA- PI4KIII β	(Godi et al., 1999)	Ampicillin
HA-PI4KIII β ^{D656A}	(Godi et al., 1999)	Ampicillin

GFP-Rab7	Addgene	Kanamycin
GFP-HA-Rab11a	Addgene	Kanamycin
GFP-TRPML V432P	Kind gift from Dr Haxiong Xu	Kanamycin
shRNAi CaBP7 (GFP)	Origene	Kanamycin
Scrambled shRNAi (GFP)	Origene	Kanamycin
pcDNA-GCaMP6s- CT	Dr Lee Haynes	Ampicillin
LAMP1-GCaMP6	<i>By sub cloning:</i> Fwd 5'atatggatccaccatggcggcccc'3 Rev 5'atatgaattcaagatggctctgatagccggcg'3 Template: LAMP1-mCherry Restriction sites: BamHI and EcoRI	Ampicillin

2.6.1. shRNAi plasmids

shRNAi control 5'- GCACTACCAGAGCTAACTCAGATAGTACT- 3'

CaBP7 shRNAi 5'- GACATGGATGGTGGTCAAGTGGACTT- 3'

Both plasmids were obtained from Origene. The sequences encode a 29-mer shRNAi under control of the human U6 promoter and a separate GFP

reporter under the control of the CMV promoter for indication of successful transfection.

shRNAi resistant mCherry-CaBP7 was generated by Dr Hannah McCue with the sense primer 5'- GACATAGAGAACAT**a**AT**t**ATGAC**a**GAGGAGGAG- 3' and antisense primer 5'- CTCCTCCTC**t**GTCAT**a**AT**t**ATGTTCTCTATGTC- 3'. This created 3 silent point mutations, which are highlighted in bold lowercase letters.

2.7. Confocal microscopy

2.7.1. Immunofluorescence (IF)

24 hours post transfection cells were washed 3 times with PBS and fixed for 6 minutes with 4% (v/v) Formaldehyde/PBS, subsequently washed 3 times with PBS and then permeabilised with 0.2% (v/v) Triton-X100/PBS for 6 minutes at RT with gentle agitation. After permeabilisation cells were again washed 3 times with PBS and twice in IF blocking buffer. Primary antibody was diluted in IF blocking buffer (Table 2.6.) and samples incubated for 1 hour at room temperature with constant agitation. Cells were washed 3 times with PBS and twice with IF blocking buffer before application of secondary Alexa Fluor antibody. Secondary antibodies were diluted in IF blocking solution (Table 2.7.) and cells incubated at RT for 1 hour. Cells were subsequently washed 3 times with PBS and coverslips allowed to air dry. Coverslips were mounted onto microscope slides using Pro Long anti-fade

glycerol mounting reagent \pm DAPI stain (Invitrogen). DAPI can be visualized with the same excitation/emission parameters as for Alexa Fluor 405.

For anti-PI4P and anti-PI (4,5) P₂ antibodies the staining protocol was modified to remove phosphate according to the manufacturer's instructions. Cells were fixed with 4% (v/v) Formaldehyde/TBS for 20 minutes at RT and washed 3 times with TBS. Cells were permeabilised with 0.5% (w/v) Saponin/ TBS at RT for 15 minutes. This was followed by 3 washes with TBS and incubation in blocking solution (10% (v/v) Goat-Serum/TBS) for 30 minutes at 37°C. Anti-PI4P/anti- PI (4,5) P₂ antibodies diluted in TBS were added to cells and samples incubated for 1 hour at 37°C. Antibody was removed and cells washed 3x with wash buffer (1% (v/v) Goat-Serum/ TBS) prior to Alexa Fluor secondary antibody incubation (30 minutes at 37°C). Secondary antibody was diluted in TBS as indicated in Table 2.7. Cells were rinsed thoroughly with distilled water, and coverslips dried completely before mounting on to microscope slides using Pro Long anti-fade glycerol mounting reagent \pm DAPI stain (Invitrogen).

2.7.2. Technical data for microscopy

Various constructs and antibodies were employed to visualize and track exogenously/endogenous protein distribution, and to determine protein co-localisation and expression levels. Details about antibodies used for immunofluorescence staining is listed in Table 2.6. Tables 2.7. and 2.8. contain confocal microscopy details such as excitation laser wavelengths and the emission wavelength ranges monitored for each fluorophore/ fluorescent

protein. All secondary Alexa Fluor dyes were obtained from Life Technologies.

Table 2.6.: Antibodies used for IF image analyses

Antibodies	Description	Company	Dilution
Anti-HA Antibody	Mouse Monoclonal	Abcam	1:500
Anti- α -tubulin (DM1A)	Mouse Monoclonal	Abcam	1:500
Anti- CaBP7	Rabbit polyclonal	Santa Cruz	1:200
Anti- CD63	Mouse Monoclonal	Abcam	1:500
Anti- LAMP1	Mouse monoclonal (H4A3)	Abcam	1:500
Anti- p230	Mouse monoclonal	Abcam	1:500
Anti- PI4KIII β	Rabbit	Millipore	1:500
LysoTracker® Red	NA	Invitrogen	50nM
Anti- GFP	Mouse monoclonal	Roche	1:500
Anti- M6PR	Rabbit polyclonal	Abcam	1:500

Anti-PI (4,5) P ₂	Mouse monoclonal	Echelon	1:200
Anti-PI4P	Mouse monoclonal	Echelon	1:200
Anti-Transferrin Receptor	Rabbit polyclonal	Abcam	1:500

Table 2.7.: Confocal microscopy parameters for secondary IF antibodies

Secondary antibodies (Alexa Fluor Dyes)	Excitation laser (nm) Emission range (nm)	Dilution
Anti- mouse/rabbit 405	405, 410 - 460	1:500
Anti- mouse/rabbit 488	488, 500 - 550	1:500
Anti- rabbit 568	594, 600 - 650	1:500
Anti- mouse 594	594, 600 - 650	1:500

Table 2.8: Confocal microscopy parameters used for fluorescent proteins

Fluorescent proteins	<i>Excitation laser (nm)/ Emission range (nm)</i>
YFP	488, 500-550
GFP	488, 500-550
mCherry	594, 610-710

2.7.3. Fixed cell confocal microscopy

Immunostained and transfected fixed cells were imaged using a Leica AOBS SP2 microscope system (Leica microsystems) with pinhole set at 1 Airy unit

and using a 63x oil immersion objective with numerical aperture (NA) of 1.4. Acquired images were exported as TIFF files and processed and analysed with ImageJ and CorelDraw X6 software packages.

2.7.4. Time-lapse confocal microscopy

Cells were plated on 35 mm glass bottom dishes (MatTek) and transfected as described previously. 24 hours post-transfection, cells were incubated on a temperature/environmentally regulated microscope stage at 37°C and 5% CO₂ and imaged using a Zeiss LSM510 (Carl Zeiss Microscopy) inverted confocal microscope with a 40x oil immersion objective with NA=1.3. EGFP and EYFP were excited using a 488nm argon ion laser and fluorescence emission detected through a 505-550nm band-pass filter. Excitation of mCherry and LysoTracker[®] DND-99 was accomplished with a 561nm Diode laser and fluorescence emission acquired with a 575-630nm band-pass filter. LysoTracker[®] Red DND-99 (Life Technologies) labelling was performed by incubating cells in media containing 50 nM of the probe at 37°C for 30 minutes. LysoTracker[®] containing media was then replaced with fresh media prior to imaging.

2.7.5. Histamine/ Ionomycin treatment

Cells plated on MatTek glass bottom dishes were transfected with LAMP1-GCaMP6 and H2B-mCherry and imaged 24 hours post-transfection. Histamine (100 µM) or Ionomycin (1 µM) containing media was added to the

dish and cells imaged with the Zeiss microscope system as previously described.

2.8. Image Analysis

2.8.1. LAMP1 and Rab5a particle counting analysis

ImageJ software was used for LAMP1/ Rab5a particle count analyses. TIFF files acquired with the Leica confocal microscope were opened with ImageJ software and converted to grey scale whereupon a region of interest (ROI) was drawn around the plasma membrane of the cell. With the ImageJ option: 'analysis - particle count', LAMP1 and Rab5a particles were counted in each individual cell. The particle count diameter range was set between 10 and infinite pixel units.

2.8.2. PI4P and PI (4,5) P₂ particle counting analysis

For PI4P/ PI (4,5) P₂ particle analysis, captured images (mCherry-CaBP7 transfected and un-transfected control cells stained with anti PI4P/PI (4,5) P₂ antibodies) were opened with ImageJ software, converted to grey scale and a ROI traced around the plasma membrane of the cell. Of 20 randomly assigned PI4P positive structures the smallest diameter was determined to be 8 pixel units and largest diameter 15 pixel units. Therefore the detection range for PI4P/PI (4,5) P₂ positive particles was set between 8 and 15 pixel units for all subsequent counting analyses.

2.8.3. Cytokinesis study analysis

Images indicating successful transfection of shRNAi plasmid or overexpressed plasmids were merged with the corresponding DAPI stained images using ImageJ. In overlay images, cells containing either abnormal nuclei number (bi-/polynucleate) or cells arrested at cytokinesis (connected by an extended intercellular bridge) were scored as abnormal against all transfected cells. Independent blind scoring gave the same results.

2.8.4. Lysosome distribution at telophase

GFP positive cells (a transfection marker present in CaBP7 shRNAi and scrambled shRNAi plasmids) at cytokinesis (determined through α -tubulin intercellular bridge staining) were analysed with ImageJ software and the 'plot profile' application. A straight line running across the intercellular bridge and extending to the edge of the nucleus of each daughter cell was drawn on p230/ LAMP1 stained sample images and the fluorescence profile along this line calculated. The area under the curve for these profiles were determined which represents the average fluorescence intensity of p230/LAMP1 in the regions of the cytoplasm and intercellular bridge directly beneath the line. Data from scrambled shRNAi samples were normalised to 100% (control) and CaBP7 shRNAi fluorescence intensity calculated as a percentage of this. Whole cell fluorescence data was also analysed from the same cells by tracing a region of interest around the plasma membrane of each cell and quantifying the average p230/LAMP1 fluorescence intensity over this area.

2.8.5. Intercellular bridge dynamics during mitosis

Time lapse videos (acquired with the Zeiss microscopy setup) of dividing cells expressing CaBP7 shRNAi (GFP as transfection marker) were timed for the appearance and disappearance of the intercellular bridge and this was compared to the same bridge dynamics in control un-transfected dividing cells on the same dish.

2.8.6. Fluorescence co-localisation analysis with JACoP

Co-localisation analyses were performed by utilizing the JACoP plug-in for ImageJ (Bolte & Cordelières, 2006). JACoP produces a Pearson's correlation coefficient (R) for each pair of images. Complete co-localisation has R=1. All images were first background subtracted and subsequently analysed with JACoP. Simultaneously Costes's randomization analysis (Costes et al., 2004) for each pair of images was performed to ensure true significance of the calculated co-localisation.

2.8.7. Calcium signalling measurements

To measure calcium signalling, HeLa cells were transfected with the calcium indicator GCaMP6 on MatTek glass bottom dishes. 24 hours post-transfection cells were imaged on the Zeiss LSM510 system under conditions as described in section 2.3.5. Excitation wavelength and the emission range were set as described in Table 2.8. for GFP. Acquisition rate varied between 4 – 6.5 seconds per frame. A ROI was drawn around the cell of interest and fluorescence intensity was measured in arbitrary units (AUs).

The GCaMP6 fluorescence measurement upon histamine treatment was calculated as the ratio of the resting fluorescence at the first time point (shown as F/F_0).

2.8.8. Lysosome activity and cell division analysis

Cells were plated onto MatTek dishes and 24 hours later, loaded with LysoTracker® Red as previously described in section 2.3.5. LysoTracker® Red containing media was then replaced with complete HeLa media. Prior to imaging, specific pharmacological agents (Table 2.9.) diluted in complete HeLa media were added to the appropriate concentration and cells imaged overnight as described in section 2.3.5. Acquisition rates varied between 6.30 – 7.50 minutes per frame.

Table 2.9.: Pharmacological inhibitors of lysosomal activity utilised in this thesis

Compound	Supplier	Working Concentration	Effect on LysoTracker® staining
Bafilomycin A1	Tocris	500 nM	Disappeared
Folimycin/Concanamycin	Abcam	100 nM	Disappeared
Glycyl-L-phenylalanine 2-naphthylamide (GPN)	Cayman chemicals	50 µM	Disappeared
Pepstatin A1	Sigma	5 µM	Unaffected
Vacuolin-1	Millipore	1 µM	Unaffected

All cells that successfully entered cytokinesis but which subsequently became binucleate or remained trapped at this point (noted as not dividing) were scored as a percentage of all dividing cells.

Chapter 3

Subcellular localisation of Calcium Binding Protein 7 (CaBP7) and its interaction with PI4KIII β

3.1. Introduction

Calcium (Ca^{2+}) is a universal intracellular second messenger that is involved in a wide range of physiological functions and cellular processes (Bootman & Roderick, 2011; Clapham, 2007). Its fundamental importance is illustrated by its range of functions including roles in fertilization, cell death, cell proliferation, gene transcription, regulation of ion channel activity, metabolism, muscle contraction, exocytosis and neuronal communication (Berridge et al., 2000; 2003). The involvement of Ca^{2+} in almost all aspects of mammalian cell biology has been made possible by the evolution of highly specific Ca^{2+} sensing proteins, which detect and respond to unique Ca^{2+} fluxes (Burgoyne & Haynes, 2010; Haeseleer, Imanishi, Sokal, Filipek, & Palczewski, 2002; McCue, Haynes, & Burgoyne, 2010a).

Calmodulin (CaM), the most intensively studied small Ca^{2+} sensing protein, is evolutionarily conserved and is present in all plant and animal cells (Chin & Means, 2000). It exhibits 100% protein identity across vertebrate species indicating an essential conserved function (Toutenhoofd & Strehler, 2000). It is a 17 kDa protein with a dumbbell like tertiary structure containing four active EF hands arranged in two pairs, one in the N-terminal lobe of the dumbbell (EF 1 and 2) and one in the C-terminal lobe (EF 3 and 4) (Lakowski, Lee, Okon, Reid, & McIntosh, 2007). The lobes of CaM are separated by a short linker sequence and on Ca^{2+} binding, the extended dumbbell structure folds compactly around this central pivot to mediate target protein binding (Chin & Means, 2000).

There are various Ca^{2+} sensing proteins evolutionarily related to CaM such as the Calcium Binding Proteins (CaBPs) (Haeseleer et al., 2000). CaBPs

are a comparatively recently discovered family of proteins which first arose with vertebrates (McCue, Haynes, & Burgoyne, 2010b), suggestive perhaps of sophisticated functions associated with complex higher organisms. CaBPs and CaM share sequence similarities (Haeseleer et al., 2000; 2002) and possess comparable EF hand arrangements (Haeseleer et al., 2002). Although CaBPs possess four EF hands, like CaM, not all of these actively bind Ca^{2+} (Haeseleer & Palczewski, 2002; Lakowski et al., 2007). In some CaBPs, the EF hand is unable to bind divalent cations, in others; the EF hand specificity is shifted from Ca^{2+} to Mg^{2+} , which appears important for physiological activity of this particular class of CaBP (CaBP1) (Wingard et al., 2005). Furthermore, in comparison to CaM, CaBPs have a four amino acid extension in the inter-lobe linker region connecting the N-terminal and C-terminal EF hand lobes that may permit CaBPs to interact with unique target proteins (Haeseleer et al., 2000).

The family of CaBPs can be divided into two subgroups regarding the pattern of inactive and active EF hand positioning. CaBPs 1-5 only possess three active and one inactive EF hand. More specifically, EF hands 1, 3 and 4 are active whereas EF hand 2 is non-functional. In comparison CaBP7 and 8 have two active and two inactive EF hands, namely the N-terminal EF hand pair (EF hand 1 and 2) are active and the C-terminal EF hand 3 and 4 are inactive (McCue, Burgoyne, & Haynes, 2011). Another key difference between these two subgroups is that CaBP7 and 8 contain a unique 38 amino acid extension at their C-terminal, which is crucial for their correct localisation and association with the Trans-Golgi Network (TGN) and plasma membrane (PM) (McCue et al., 2009). This C-terminal extension contains a

specialised transmembrane domain, which is characteristic of the tail-anchored class of membrane proteins. These proteins are unique as they are inserted post-translationally into cellular membranes (Hradsky et al., 2011). CaBP7 and 8 display the classic tail-anchor type II membrane protein topology, with the majority of the protein, including the functional C-terminal EF hands, oriented toward the cytoplasm, a single transmembrane domain and short luminal C-terminal tail (McCue et al., 2011).

Additional phylogenetic studies further support the distinction between the two CaBP subgroups (Haynes, McCue, & Burgoyne, 2012). Studies revealed that CaBPs 1-5 form a different branch from CaBP7 and 8, and it has been speculated that these differences may be of functional relevance (McCue, Haynes, & Burgoyne, 2010b). CaBPs 1-5 have been studied for their functions in regulation of voltage-gated calcium channels (VGCCs) including the high voltage activated P/Q- (Lee et al., 2000) and L-type channels (Zhou et al., 2005; Cui et al., 2007; Yang et al., 2006a; Haeseleer et al., 2004), in addition to the inositol 1,4,5 trisphosphate receptor (IP₃R, Haynes et al., 2004), and transient receptor potential channel-5 (TRPC5, Kinoshita-Kawada et al., 2005).

CaBP7 and 8 on the other hand have been shown to negatively regulate PI4KIII β at low or resting Ca²⁺ levels (Mikhaylova et al., 2009). PI4KIII β is a soluble enzyme that can be recruited to the TGN where it phosphorylates phosphatidylinositol (PI) to phosphatidylinositol -4 phosphate (PI4P) (Clayton, Minogue, & Waugh, 2013). PI4P production at the TGN has an essential function in the biogenesis of secretory vesicles that exit from this organelle and transport cargo to the PM (Burgoyne & Haynes, 2010; Haynes

et al., 2005). Overexpression of CaBP7 and 8 led to the reduction of the PI4KIII β product PI4P and in turn inhibited TGN to PM trafficking in COS-7 cells (Mikhaylova et al., 2009). Upon an increase in cytosolic Ca²⁺ above basal concentrations, neuronal calcium sensor 1 (NCS-1) preferentially binds to PI4KIII β which instead activates the kinase leading to an increase in PI4P levels, and stimulation of TGN to PM trafficking (Burgoyne & Haynes, 2010; Mikhaylova et al., 2009). To date PI4KIII β is the only known interaction partner for CaBP7 and 8.

The CaBP family have been defined mainly as neuronal specific Ca²⁺ sensors (Haeseleer et al., 2000), and research has focused on their functions in neurons. Nonetheless, the human protein atlas project database suggests all CaBPs to be more ubiquitously expressed in both neuronal and non-neuronal tissues (www.proteinatlas.org).

Consistent with a more ubiquitous pattern of expression, a recent high throughput RNAi screen using HeLa cells to investigate proteins important during mitotic cell division identified CaBP7 as an important regulator of this critical process. Detailed analysis showed that CaBP7 knockdown caused an increased binucleate cell rate, indicative of failed cellular separation and suggesting a role for CaBP7 in the final stage of mammalian cell division, cytokinesis (Neumann et al., 2010). This further broadens the importance of CaBP7 expression to dividing cells rather than restricting its expression to the non-mitotic cells of the adult mammalian nervous system. The aim of this chapter was to expand the current knowledge of CaBP7 function by investigating its subcellular localisation in HeLa cells and its interaction with PI4KIII β .

3.2. Results

3.2.1. Subcellular localisation of CaBP7 in HeLa cells

In the RNAi screen of Neumann *et al.* HeLa cells were used suggesting that CaBP7 is expressed in this model cell line. HeLa cells are cervical cancer cells, which have been used in scientific research since 1950 (Callaway, 2013). In order to employ HeLa cells to further study CaBP7, it was important that the expression of CaBP7 in these cells was confirmed. Reverse transcription PCR (RT-PCR) with primers specific for the human CaBP7 sequence was performed on cDNA template generated from HeLa cell total RNA primed with oligo-dT₍₁₅₎ primers. This analysis generated a single PCR product between 0.6- 0.8 kb, which is consistent with the expected size for the CaBP7 cDNA nucleotide sequence (645bp). As a control CaBP7 sequence was also generated with the same primers from the mCherry-CaBP7 construct (Figure 3.1 A). To expand on this expression analysis, the presence of CaBP7 protein in HeLa cell lysates was verified by Western blotting using a CaBP7 specific antibody (Figure 3.1 B). Blots revealed an immunoreactive band at approximately 30 kDa which is slightly higher than the predicted molecular mass of CaBP7 of ~24 kDa.

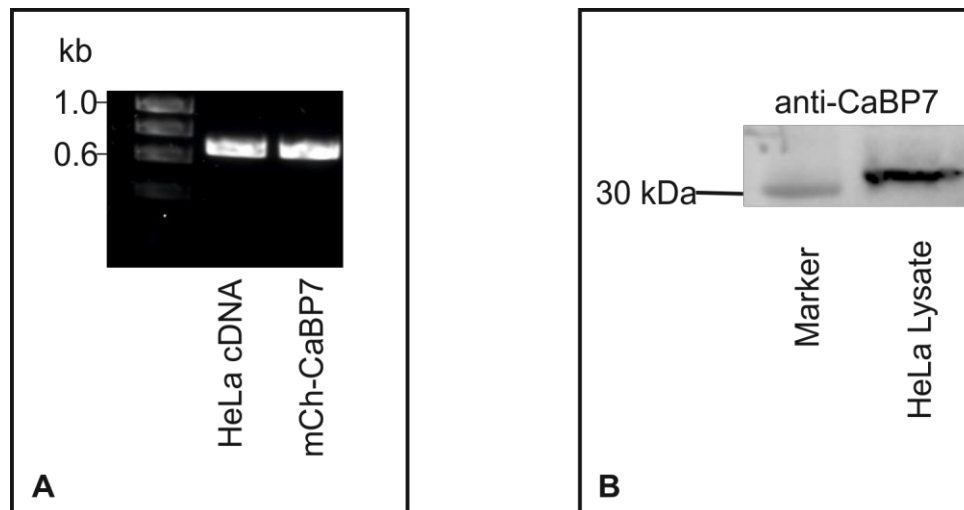


Figure 3.1. Expression of CaBP7 gene and endogenous CaBP7 protein in HeLa cells. A) RT-PCR of the CaBP7 sequence from HeLa cell cDNA with CaBP7 specific primers. A sequence verified mCherry-CaBP7 expression plasmid was also included as a template in this PCR screen as a positive control (mCherry-CaBP7); B) Western blot on HeLa cell lysate with CaBP7 specific antibody.

Since the CaBP7 immunoreactive band on the Western blot ran slightly higher than expected at around 30 kDa (Figure 3.1 B), specificity of the antibody required to be examined. CaBP7 knockdown experiments also failed to show a reduction with this antibody via western blotting, even though a phenotype was apparent upon CaBP7 knockdown. Therefore the specificity of this antibody for western blotting is questionable. However the CaBP7 antibody was specific for immunofluorescence experiments. HeLa cells transfected with exogenous mCherry- CaBP7 were stained with the antibody and processed for immunofluorescence. CaBP7 antibody – if specific to CaBP7- should stain all exogenously expressed mCherry- CaBP7, which can be seen in Figure 3.2. A and B in the merged images, suggesting that the antibody is specifically detecting CaBP7. The correct expression of the

mCherry-CaBP7 construct was also tested by western blotting for its precise protein size (mCherry tag 28 kDa + CaBP7 24 kDa = 52 kDa) (Figure 3.2 C).

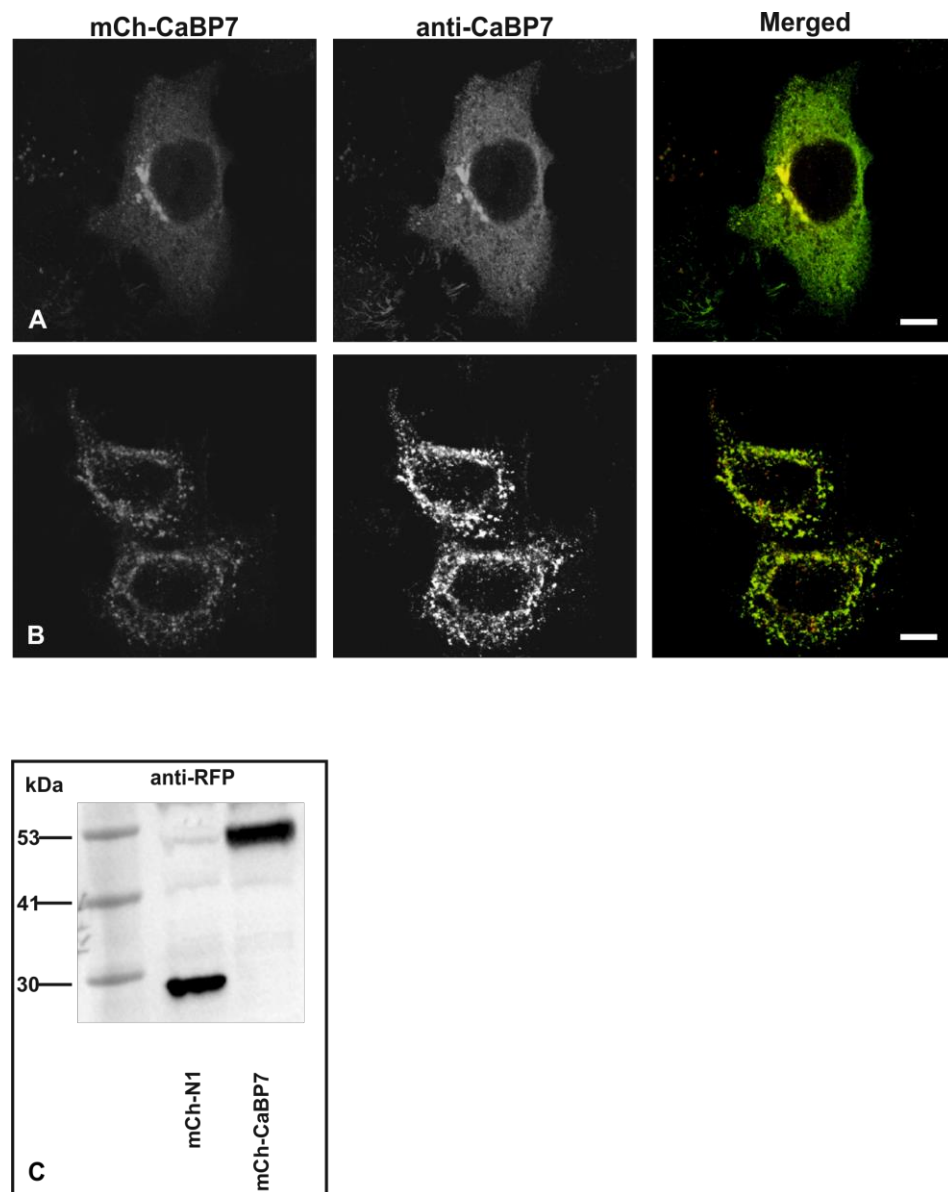


Figure 3.2. Validating CaBP7 antibody in HeLa cells expressing mCherry-CaBP7. A and B) Examples of HeLa cells expressing exogenous mCherry-CaBP7 (red) stained with CaBP7 antibody (green) to validate the specificity of the antibody. Regions of co-localisation appear yellow in the overlay images. Scale bars = 10 µm. C) HeLa cell lysates expressing control mCherry-N1 and exogenous CaBP7 (mCherry-CaBP7) blotted with Red fluorescent protein (RFP) antibody to detect mCherry.

After validating the specificity of the antibody, HeLa cells were immunostained with anti- CaBP7 to detect the endogenous expression pattern of CaBP7. Cells were fixed and co-stained with anti- tubulin antibody (microtubule network marker) and DAPI (nuclear marker) to accurately evaluate the distribution of CaBP7. Endogenous CaBP7 displays a varying and heterogeneous distribution in cell populations. In some cells there is a predominantly perinuclear localisation (Figure 3.3. A) and in others there is a more widely distributed pattern to vesicular structures located throughout the cytoplasm (Figure 3.3. B). The perinuclear localisation represents the TGN as CaBP7 has known functions on this organelle (Burgoyne & Haynes, 2010; Mikhaylova et al., 2009). The vesicular and disperse distribution was unusual and merited further investigation.

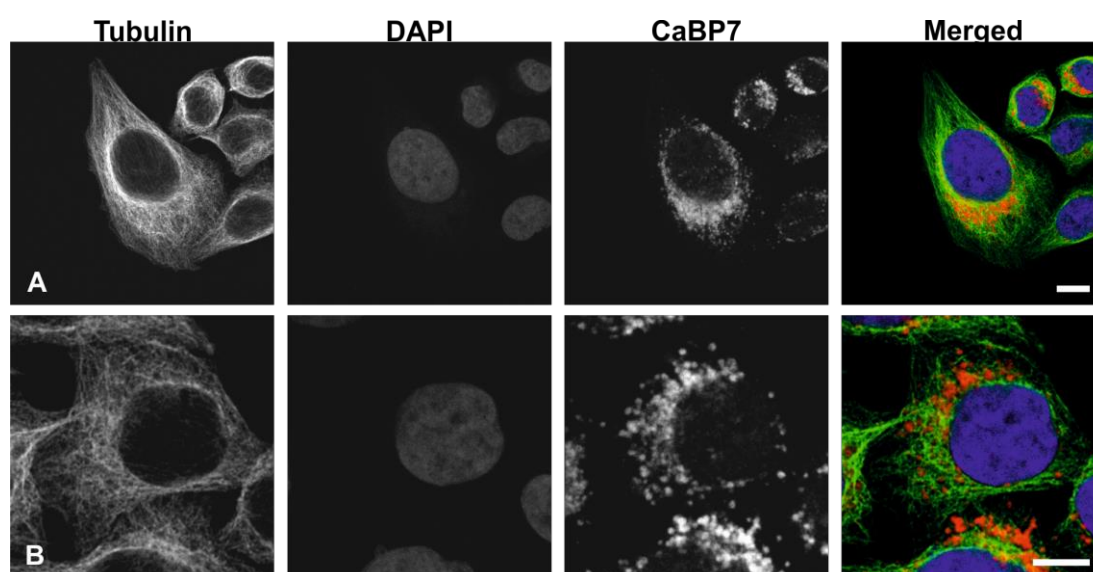
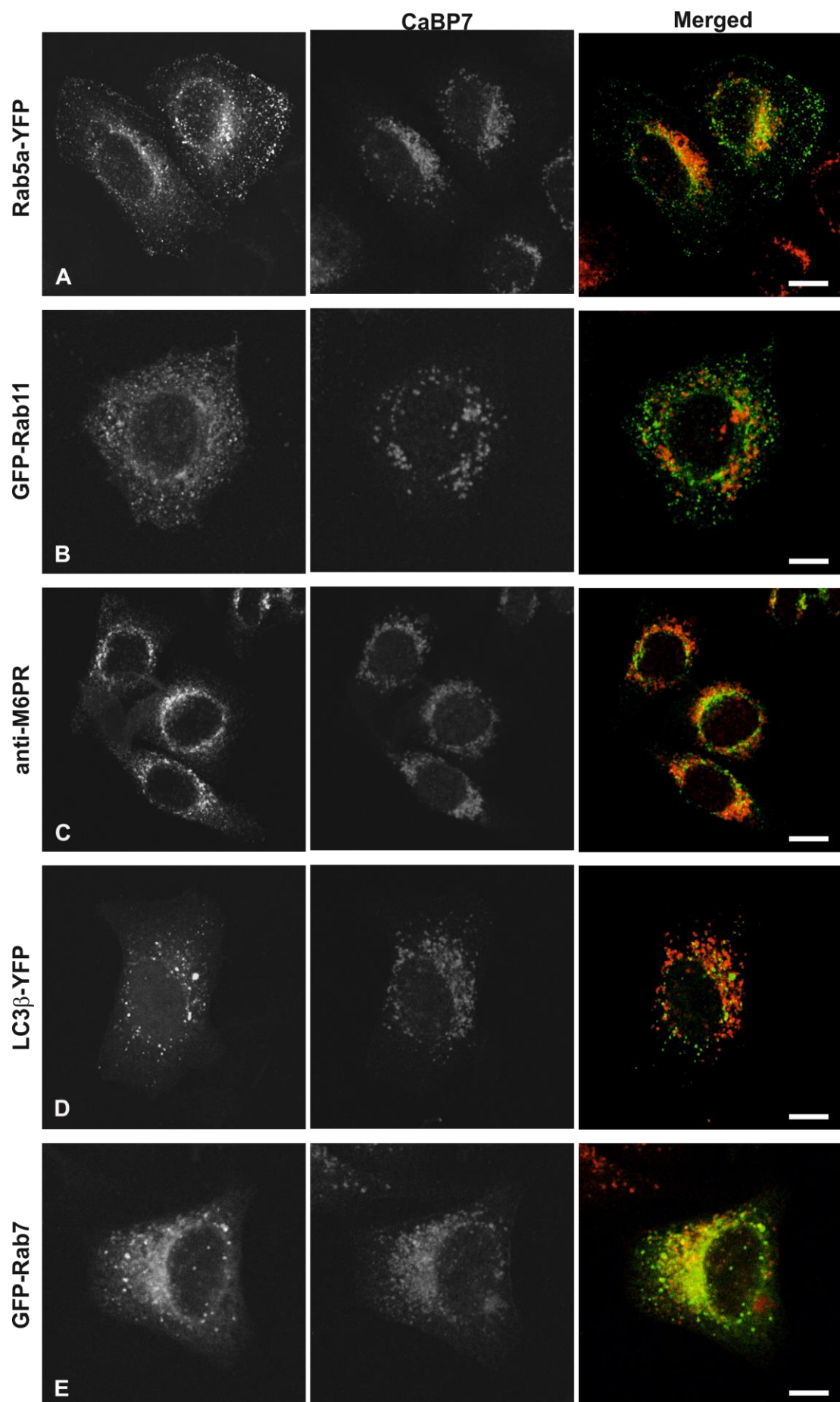


Figure 3.3. HeLa cells immunostained with CaBP7 specific antibody. Examples of HeLa cells stained with anti-tubulin (green), nuclear marker DAPI (blue) and the CaBP7 specific antibody (red). Scale bars = 10µm.

3.2.2. Endogenous CaBP7 co-localisation studies with subcellular markers

In a proportion of HeLa cells, CaBP7 displayed a vesicular localisation throughout the cytoplasm (Figure 3.3. B). To further identify CaBP7 specific structures cells were co-stained or transfected with various intracellular compartment markers or constructs. Evaluating the extent of co-localisation of CaBP7 with different subcellular markers allowed more precise delineation of CaBP7 localisation. Extent of co-localisation was determined by the amount of overlap (yellow appearance in overlay images) between CaBP7 staining (red) and the subcellular markers (green). Endogenous CaBP7 showed only partial co-localisation with the early endosome marker Rab5a and recycling endosomal marker Rab11 (Figure 3.4. A, B). Similarly limited co-localisation was noticeable with mannose 6-phosphate receptor (M6PR), which cycles between the TGN and endosomes but which is completely absent from compartments of the late endocytic pathway. There was also a lack of co-localisation with the autophagosomal marker LC3 β (Figure 3.4. C, D). More extensive co-localisation was observed with the late endosome/lysosome marker Rab7 (Figure 3.4. E) and an almost complete overlap was evident with the lysosomal enriched proteins CD63 and LAMP1 as well as the acidotropic probe LysoTracker[®] (Figure 3.4. F- H), suggesting extensive association of CaBP7 with late endosomes/lysosomes. Collectively, these co-localisation data highlight an absence of CaBP7 from compartments of the early endosomal and autophagic pathways and enrichment in acidic late endosomal compartments consistent with localisation to late endosomes/lysosomes. In addition to the documented

localisation of CaBP7 to the TGN and PM the results presented here clarify the observed yet unexplained localisation of CaBP7 to punctate structures in a proportion of HeLa cells (Rajamanoharan et al., 2015).



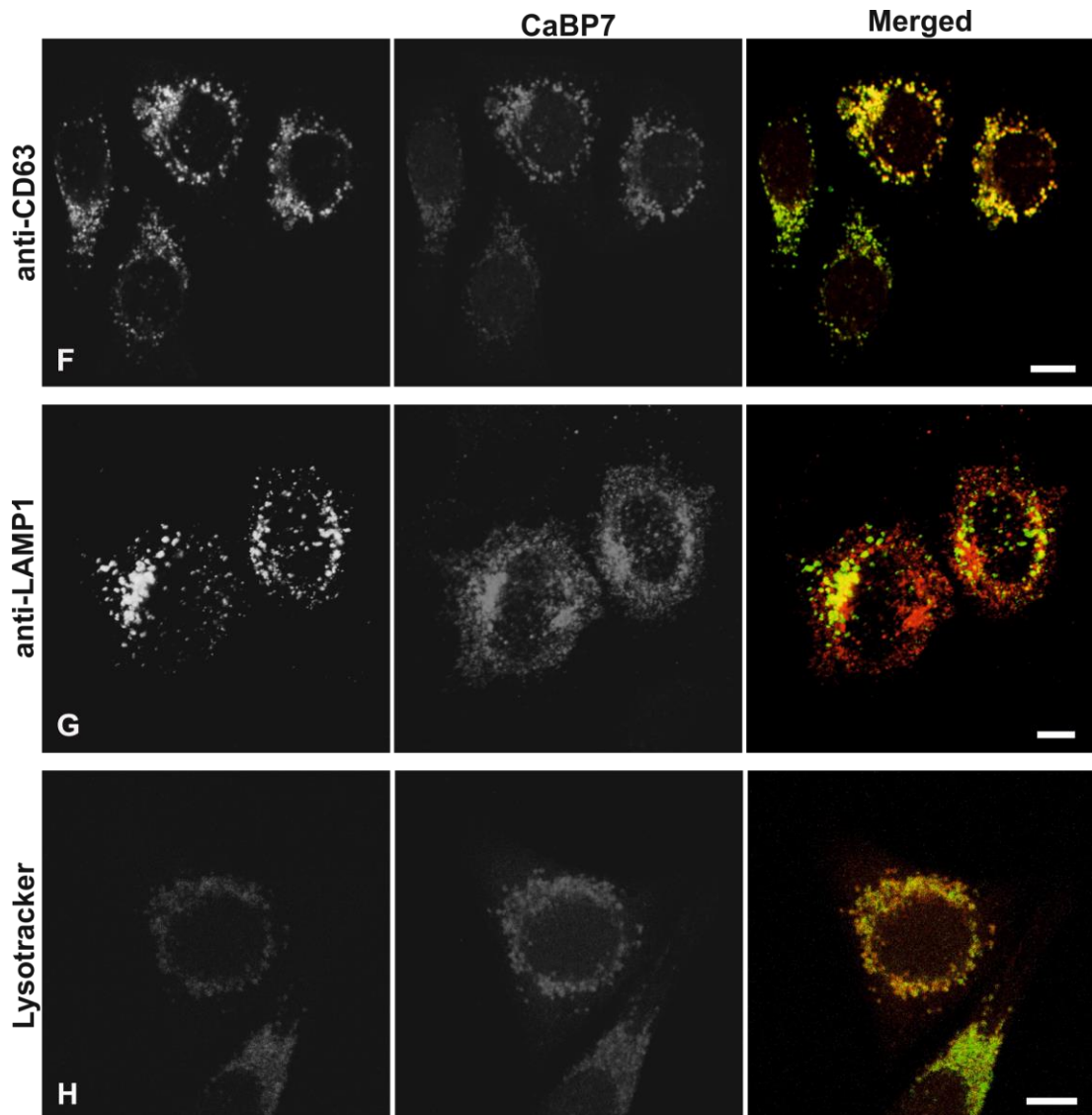


Figure 3.4. Co-localisation studies of endogenous CaBP7 with various intracellular compartment markers. HeLa cells stained with anti- CaBP7 (red) co-transfected or stained with specific intracellular markers (green). Regions of co-localisation appear yellow in the overlay images. A) Early endosome marker Rab5a; B) recycling endosome marker Rab11; C) marker for TGN and endosomes M6PR; D) autophagosome marker LC3 β ; E) late endosome marker Rab7; F) late endosome/ lysosome marker CD63; G) lysosome marker LAMP1 and H) stained with the acidotropic probe LysoTracker[®]. Scale bars = 10 μ m.

3.2.3. CaBP7 function on lysosomes

PI4KIII β has been studied extensively and identified for its important roles in maintaining the structure and function of the Golgi apparatus (Godi et al., 1999b; 2004; Balla and Balla, 2006; Faulhammer et al., 2007). In a more recent study by Sridhar *et al.*, a biochemically distinct pool of PI4KIII β was identified that associated with lysosomes and which exerted a functional role in maintaining lysosomal identity through controlling lysosomal content sorting and biogenesis of transport carriers (Sridhar et al., 2013). In this study the down regulation of PI4KIII β [using RNA interference (RNAi)] induced lysosomal clustering in a perinuclear region and the loss of normal dispersed lysosomal distribution.

Building on these observations and data showing that CaBP7 negatively regulates PI4KIII β at the TGN (Burgoyne & Haynes, 2010; Mikhaylova et al., 2009) the question arose whether CaBP7, might also regulate this novel lysosomal pool of PI4KIII β . To address this question, the lysosome-clustering assay (as employed by Sridhar et al., 2013) was used. If CaBP7 is indeed regulating the lysosomal pool of PI4KIII β , overexpression of CaBP7, a known inhibitor of PI4KIII β , would be expected to mimic the PI4KIII β silencing condition as seen by Sridhar *et al.* (Sridhar et al., 2013).

Initially, the normal distribution of lysosomes was confirmed by transiently expressing LAMP1-YFP in HeLa cells. LAMP1-YFP displayed a typical localisation to punctate structures dispersed throughout the cytoplasm (Figure 3.5.). Next, the lysosome clustering assay was performed. All numerical and statistical data for the lysosome clustering assay can be found in Table 3.1.

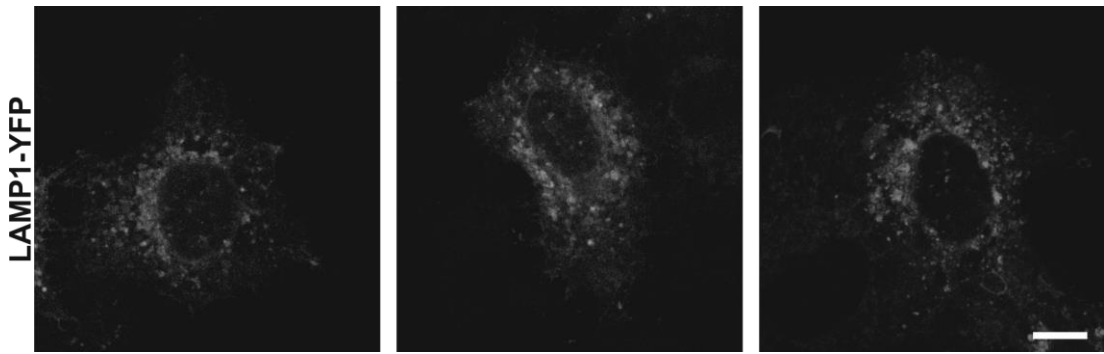


Figure 3.5. LAMP1-YFP distribution in HeLa cells. Example images of HeLa cells expressing exogenous lysosomal associated membrane protein -1 (LAMP1-YFP). Scale bars = 10 μ m.

Calcium binding protein 4 (CaBP4) was included in the assay as a negative control, as this is a cytosolic protein related to CaBP7 but having no known interaction with PI4KIII β . Lysosome distribution after CaBP4 overexpression displayed the same disperse distribution (Figure 3.6. A) observed in control LAMP1-YFP (Figure 3.5.) expressing cells. PI4KIII β ^{D656A}, a kinase- inactive version of the enzyme (Godi et al., 1999) was included as a positive control to mimic the knockdown of PI4KIII β . Indeed PI4KIII β ^{D656A} generated visible lysosome clustering (Figure 3.6. B). As expected, CaBP7 overexpression also changed LAMP1-YFP localisation, eliciting a noticeable clustering in the perinuclear region (Figure 3.6. C), which was comparable to PI4KIII β silencing (Sridhar et al., 2013) or PI4KIII β ^{D656A} overexpression (Figure 3.6 B). Quantification of lysosome numbers under the various conditions tested confirmed the observed clustering results (Figure 3.8.). These results led to the conclusion that CaBP7 was possibly regulating the lysosomal pool of PI4KIII β . Published data indicates that CaBP7 interacts with PI4KIII β on the TGN at resting Ca²⁺ levels (Mikhaylova et al., 2009). Therefore the novel interaction between lysosome resident PI4KIII β and CaBP7 was investigated

for Ca^{2+} binding dependency. For this analysis specific CaBP7 EF hand mutants were utilised. Three constructs with different EF hand motif mutations were tested: either EF hand 1 (EF 1) or EF hand 2 (EF 2) were individually defective in Ca^{2+} binding or both functional EF hands were inactive (EF 1+2). These mutants were generated by introducing single-point mutations to change glutamic acid to glutamine at position 12 in the Ca^{2+} -binding loop, an established method to abolish Ca^{2+} binding capabilities of EF hand motifs (da Silva, Kendrick-Jones, & Reinach, 1995). All mutants tested exhibited the same lysosome clustering phenotype (Figure 3.7. and 3.8.) observed with wild type CaBP7 overexpression (Figure 3.6 C). These results suggest that the inhibitory regulation of PI4KIII β on lysosomes by CaBP7 can occur independently of Ca^{2+} binding consistent with the known mode of regulation observed at the TGN.

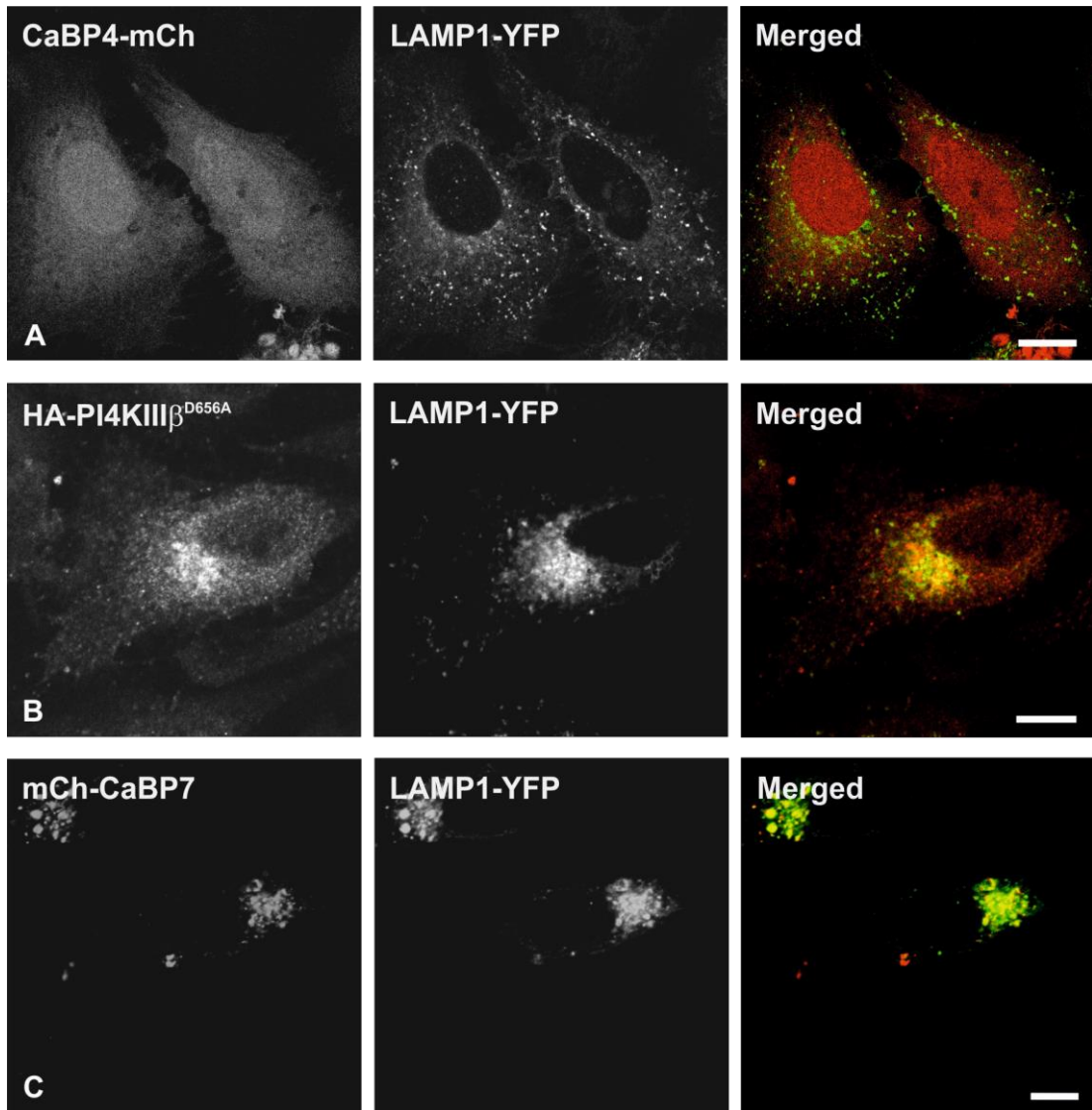


Figure 3.6. CaBP7 and PI4KIII β ^{D656A} cause lysosome clustering. HeLa cells co-expressing CaBP4, PI4KIII β ^{D656A}, CaBP7 with LAMP1-YFP (green) were assessed for lysosomal clustering. A) CaBP4-mCherry (red, negative control) and LAMP1-YFP. B) HA-PI4KIII β ^{D656A} (positive control, red) and LAMP1-YFP, C) mCherry-CaBP7 (red) and LAMP1-YFP. Regions of co-localisation appear yellow in the overlay images. Scale bars = 10 μ m.

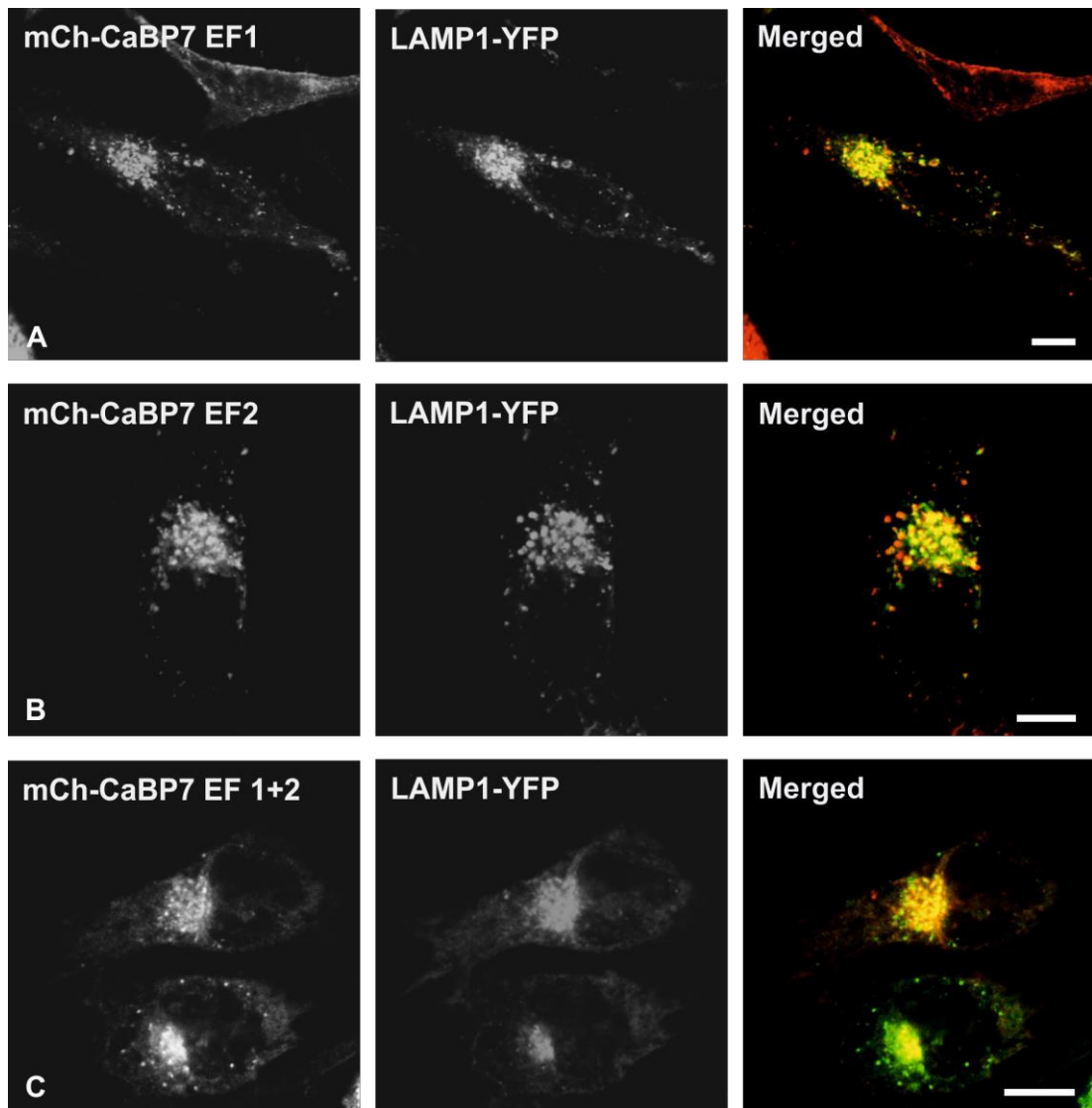


Figure 3.7. Lysosome clustering is not dependent of Ca^{2+} binding activity of CaBP7. HeLa cells expressing CaBP7 EF hand mutants (red) and LAMP1-YFP (green) were assessed for lysosomal clustering. A) mCherry-CaBP7 EF hand 1 mutant with LAMP1-YFP, B) mCherry-CaBP7 EF hand 2 mutant with LAMP1-YFP, C) mCherry-CaBP7 EF hand 1+2 mutant (double mutant) with LAMP1-YFP. Regions of co-localisation appear yellow in the overlay images. Scale bars = 10 μm

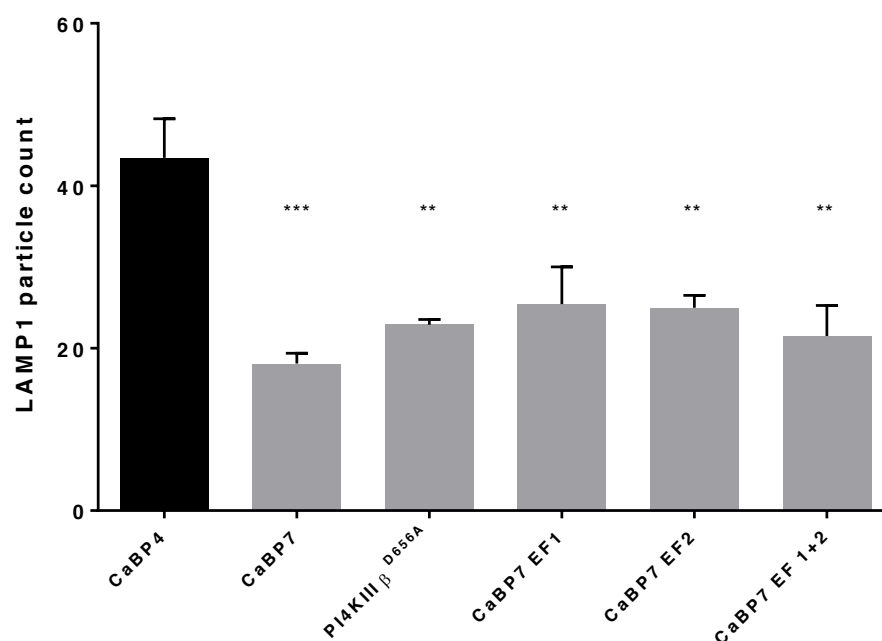


Figure 3.8. Quantification of LAMP1 particle count. Histogram showing the average LAMP1 particle counts \pm SEM upon expression of different exogenous proteins. One-way ANOVA comparing each data set to CaBP4 (control) was performed (** $p < 0.01$, *** $p < 0.001$).

Construct	LAMP1 particle count	\pm SEM	Fold reduction compared to control	Cells analysed	Number of independent experiments
CaBP4	43	4.8	1.0	109	3
CaBP7	18	1.3	2.4	102	3
PI4KIII β D656A	23	0.6	1.9	97	3
CaBP7 EF1	25	4.6	1.7	86	3
CaBP7 EF2	25	1.6	1.7	120	3
CaBP7 EF 1+2	21	3.8	2.0	123	3

Table 3.1. Numerical and statistical data for lysosome clustering assay.

3.2.4. CaBP7 does not influence early endosome trafficking

As the previous results did not provide any evidence for a direct interaction between the lysosome based PI4KIII β and CaBP7 proteins, further controls were performed to ensure the lysosome specific influence of overexpressed CaBP7. To exclude the possibility that CaBP7 influenced the early endosomal trafficking pathway, the same clustering assay was employed but where Rab5a (early endosome marker) distribution was assayed instead of LAMP1. Rab5a exhibited the same randomly dispersed vesicular distribution in both CaBP4 and CaBP7 overexpressing cells (Figure 3.9. A and B). Quantification of these images indicated no significant difference in particle count (Figure 3.9. C) confirming that CaBP7 does not interfere with the distribution of early endosomal compartments that are directly upstream of late endosomes/lysosomes.

To further support the lysosome specific effect by CaBP7, M6PR distribution was similarly assayed. M6PRs cycle between PM and endosomes and from endosomes to TGN, but are effectively excluded from lysosomes. No obvious change in either morphology or localisation of M6PR positive organelles was detectable upon CaBP7 overexpression compared to CaBP4 overexpression (control) (Figure 3.9. D and E). Since CaBP7 failed to induce any changes in the morphology or distribution of the early endosomal markers Rab5a and M6PR, it can be concluded that CaBP7 overexpression specifically influences lysosome distribution. This is possibly due to the interaction of CaBP7 with the lysosomal pool of PI4KIII β and inhibition of its kinase activity.

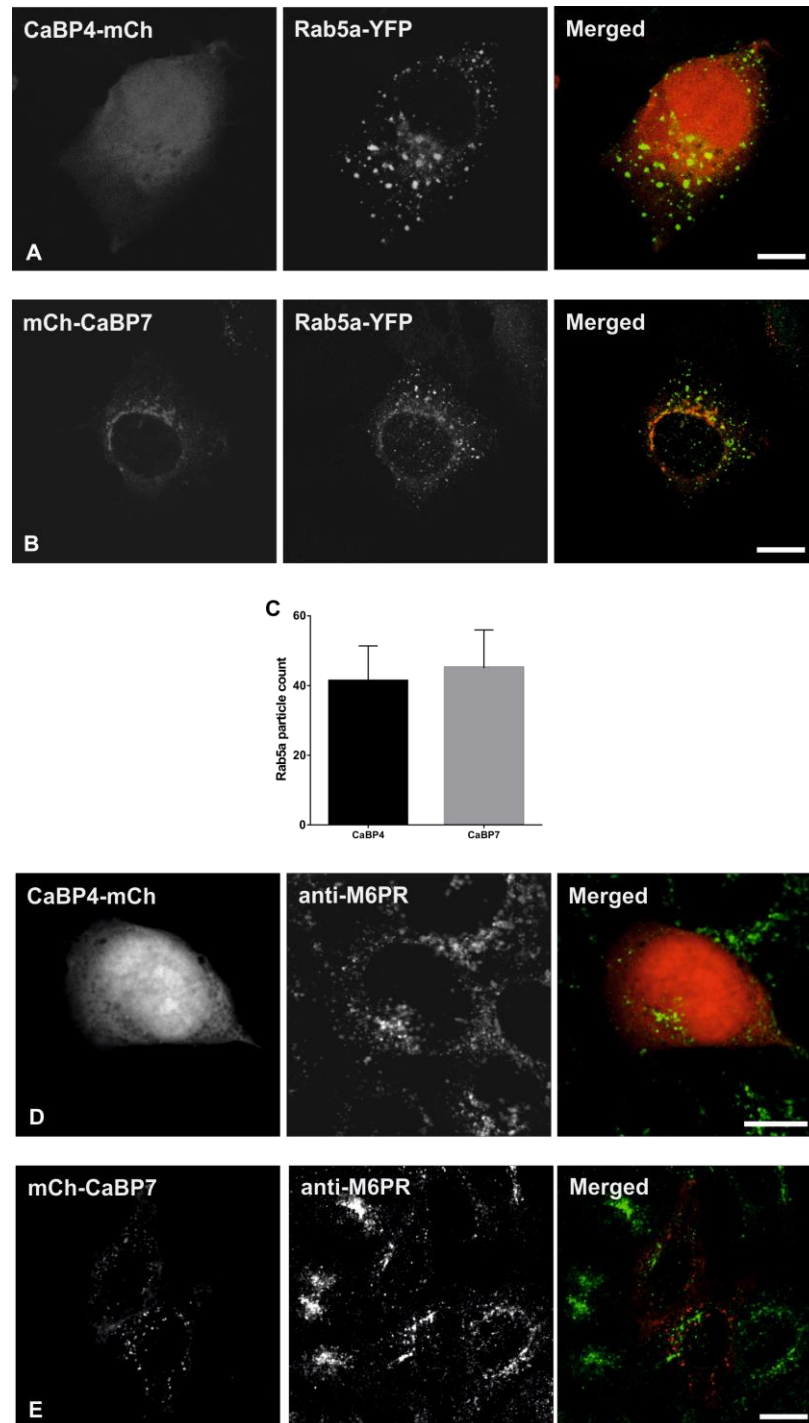


Figure 3.9. CaBP7 does not influence early endosomal pathways. HeLa cells expressing CaBP4 and CaBP7 alone co-transfected with Rab5a-YFP or immunostained for endogenous M6PR were assessed for the morphology and distribution of early endosomal compartments. A) CaBP4 (red) and Rab5a (green), B) CaBP7 (red) and Rab5a (green), C) Histogram of Rab5a particle count quantification \pm SEM from 3 independent experiments, D) CaBP4 (red) and M6PR (green), E) CaBP7 (red) and M6PR (green). Scale bars = 10 μ m.

3.2.5. CaBP7 influences PI4P levels

Collectively, these lysosomal clustering analyses suggested a possible role for CaBP7 in negatively regulating lysosome specific PI4KIII β . To provide additional evidence of PI4KIII β inhibition by CaBP7, levels of its product PI4P was examined. The enzyme PI4KIII β phosphorylates phosphatidylinositol (PI) to generate PI4P (Clayton et al., 2013). Therefore the inhibition of this enzyme should ultimately result in the reduction of cellular PI4P levels. CaBP7 overexpressing and un-transfected control cells were immunostained with a PI4P specific antibody and the number and intensity of PI4P positive particles determined (Figure 3.10. A). The representative images and the quantification (Figure 3.10. A and C) indicate a global reduction of PI4P staining in the CaBP7 expressing cells (58% reduction) compared to control cells, supporting that CaBP7 efficiently inhibits PI4KIII β . The reduction in PI4P levels is significant considering that there are at least three other known PI4K enzyme subtypes that are able to generate PI4P from PI (T. Balla, 2013).

Since PI4P is a major precursor for PI (4,5) P₂ (Tan & Brill, 2014), it was essential to additionally investigate whether CaBP7 also modulated levels of this most abundant phosphoinositide signalling species which might explain the effects on lysosomal clustering. Although a small reduction of PI (4,5) P₂ was observed in CaBP7 overexpressing cells compared to control cells, it was not statistically significant (Figure 3.10. B and C). Therefore CaBP7 expression in these assays appears not to influence PI (4,5) P₂ driven cellular events.

To independently verify the reduction of PI4P levels in CaBP7 overexpressing cells a dot-blot analysis was employed. Lipids extracted from CaBP7 overexpressing cells and control cells expressing mCherry-N1 were evaluated for their PI4P content via Western blotting of PIP-Strip™ membranes. No difference could be detected between the two samples (Figure 3.10. D), which is most likely explained by limited transfection efficiency in these experiments.

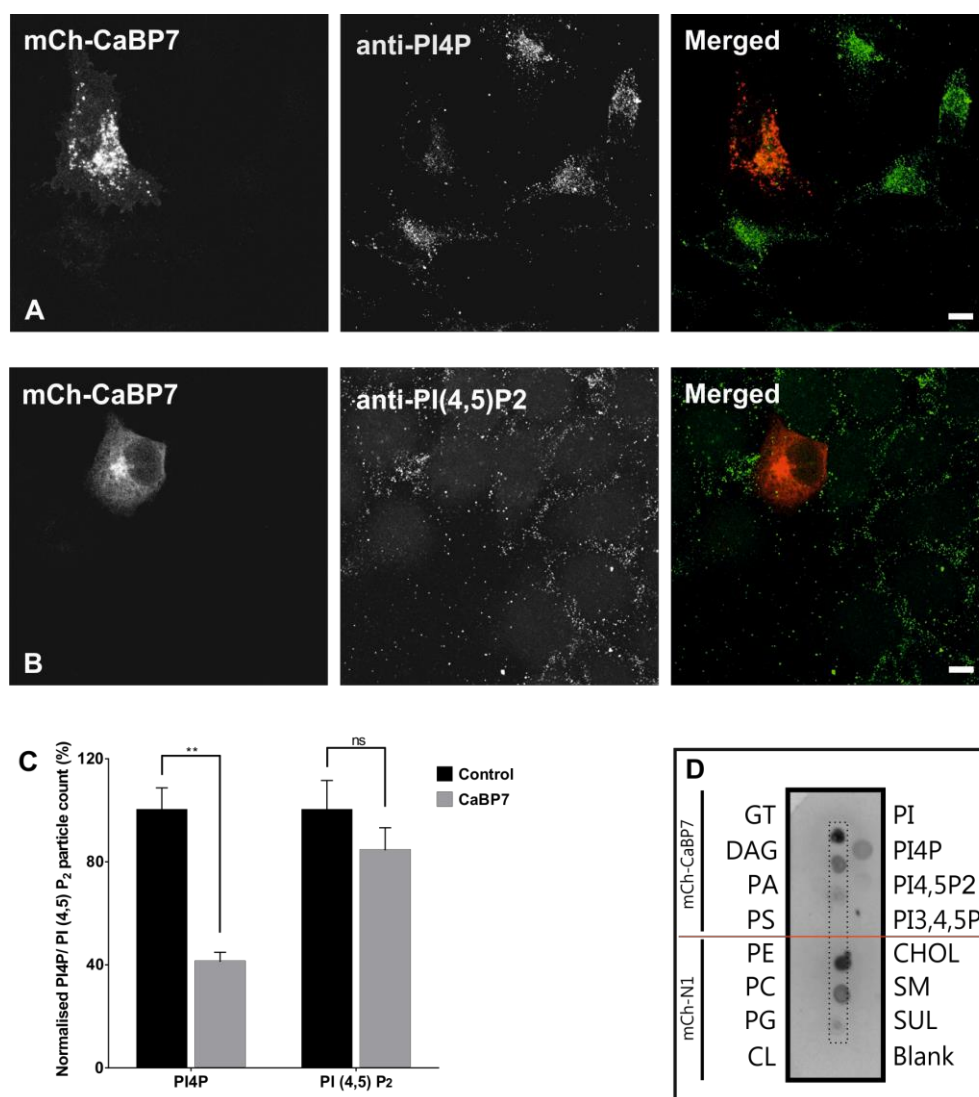


Figure 3.10. CaBP7 reduces PI4P but not PI (4,5) P₂ levels. A and B) Cells were transfected with CaBP7 (red) and stained with A) anti-PI4P (green), B) anti- PI (4,5) P₂ (green), C) Histogram of PI4P/ PI (4,5) P₂ positive particles quantification in CaBP7 expressing and un-transfected cells (control), all data plotted \pm SEM, unpaired t-test was performed (**p<0.01), D) Lipid dot blot analysis of cellular PI4P levels in CaBP7 and mCherry-N1 (control) expressing cells. Samples of extracted cellular lipids were serially diluted and equal volumes spotted on to the centre of a PIP-Strip™ membrane (indicated by the dotted box in the centre of the membrane). Undiluted (top spot) and 2x and 4x dilutions (lower spots) were dried on the membrane and probed for PI4P presence by GST- tagged PI4P-Grip protein followed by anti-GST incubation. Control PI4P lipid dot is visible on the right side, second row. The positions of other lipid standards present on the membrane are indicated along the left and right hand sides of the image. Abbreviations can be found in material and methods.

3.3. Discussion

The calcium binding proteins are relatively recently discovered proteins (Haeseleer et al., 2000), which evolved along with vertebrates (McCue, Haynes, & Burgoyne, 2010b). Some of the CaBPs have well documented roles in the regulation of Ca^{2+} channels and in the coordination of neuronal gene transcription (Dieterich et al., 2008), however, for other members of the family, including CaBP7 and 8, there is a relative paucity in functional information. CaBPs 1-5 are mainly expressed in neurons, and sensory tissues including the retina and inner ear (Di Donato, Auer, Duroure, & Del Bene, 2013; Haeseleer et al., 2002) and have been shown to bind to and modulate various CaM targets including VGCC (A. Lee et al., 2002; P. S. Yang et al., 2006; H. Zhou et al., 2005; 2004), transient receptor potential (TRP) channels (Kinoshita-Kawada et al., 2005), and IP_3 Receptors (Kasri et al., 2004; C. Li et al., 2013; Oz et al., 2013). Although CaBPs and CaM share common target interactions the targeting mechanisms, interaction motifs and modes of regulation in these instances have been found to be highly specific and non-redundant. CaBPs therefore are likely to exert unique, non-redundant, functions (Oz et al., 2013; Rieke et al., 2008; P. S. Yang et al., 2014).

CaBP7 and 8 form a distinct subgroup of CaBPs. Their unique domain structure and EF hand activation pattern, suggests that they may have novel functional roles in vertebrates (McCue, Haynes, & Burgoyne, 2010a). Furthermore, in contrast to other CaBPs that regulate various types of Ca^{2+} channels, CaBP7 and 8 have thus far only been shown to interact with the Golgi associated lipid modifying enzyme PI4KIII β (Mikhaylova et al., 2009).

The particular interest in this chapter is centred on CaBP7, as this protein has been identified as the only calcium binding protein to play a key role in cell division, the biological process which is the focus of further work in this thesis (Neumann et al., 2010).

After confirmation of CaBP7 gene and protein expression in HeLa cells, the heterogeneous localisation pattern of CaBP7 was investigated. CaBP7 displays, in some cells, a prominent perinuclear distribution whereas in others a more disperse vesicular localisation is observed (Figure 3.3.). Through co-localisation studies it was possible to confirm previous observations that CaBP7 positive vesicular structures exhibit extensive co-localisation with lysosomal markers (Figure 3.4.) and only limited or no co-localisation with early endosomal markers (Rab5), recycling endosomes (Rab11, M6PR) and autophagosomes (LC3 β). Three different markers including CD63, LAMP1 and LysoTracker® were employed to tightly pinpoint the co-localisation of CaBP7 with lysosomes. It should be noted that even though these markers are enriched on lysosomes they also appear at significant levels on late endosomes and distinguishing between these two organelle populations is extremely challenging using fluorescence based microscopy approaches. LAMP1 along with CD63 are the most abundant lysosomal membrane proteins (Eskelinen et al., 2003; Saftig & Klumperman, 2009). The extensive co-localisation of CaBP7 with lysosome membrane proteins strongly suggests CaBP7 to have a lysosome resident function considering its known topology whereby its functional domains are exposed to the cytoplasm (McCue et al., 2011). CaBP7 is known to display a type II membrane protein orientation (McCue et al., 2011). The targeting properties

of membrane proteins are usually encoded in the transmembrane domain based on hydrophobicity, length and lipid binding (Hradsky et al., 2011). Studies have shown membrane proteins with 15-17aa TMD to be localised to the endoplasmic reticulum, whereas proteins containing a TMD over 20aa are inserted into the TGN, endosome/ lysosomes or PM (Sprong, van der Sluijs, & van Meer, 2001). CaBP7's hydrophobicity score of 48.8 – 52.7 (Hradsky et al., 2011) plus its 23aa long TMD as predicted by HMMTOP (Prediction of transmembrane helices and topology of proteins) and ProtScale software (Hradsky et al., 2011; McCue et al., 2009) is therefore entirely consistent with localisation to lysosomes. Hradsky *et al.* revealed that CaBP8 (and most likely CaBP7) is targeted to the TGN via the TRC40/Asna1 chaperone complex. The targeting signal and trafficking pathway of CaBP7 to lysosomes requires more research to decipher specific sequences or motifs that might permit this localisation. Typical transport of lysosomal membrane proteins involves a complex interplay of luminal and cytosolic signals and motifs (Braulke & Bonifacino, 2009) with either a direct Mannose-6 phosphate dependent or independent mechanism (Saftig & Klumperman, 2009).

CaBP7 localisation to the Golgi and PM has been studied in detail (Burgoyne & Haynes, 2010; McCue et al., 2011), but its localisation to lysosomes is novel (Rajamanoharan et al., 2015). Specific localisation of proteins tightly links with their function, hence CaBP7 activity on lysosomes required further investigation. CaBP7's function on the TGN is to inhibit the Golgi resident PI4KIII β enzyme at resting Ca²⁺ levels, which in turn reduces PI4P levels and therefore TGN to PM trafficking is down regulated. This has been speculated

to represent a mechanism whereby localised Ca^{2+} signalling can control specific vesicular transport pathways at the TGN (Burgoyne & Haynes, 2010; Mikhaylova et al., 2009).

Sridhar *et al.* published evidence of a biochemically distinct pool of PI4KIII β on lysosomes (Sridhar et al., 2013). In their study they demonstrated that the knockdown of PI4KIII β or the overexpression of the kinase inactive version of the enzyme (PI4KIII β^{D656A}) caused lysosome clustering. Following up on this, in the latter part of this chapter the regulation of PI4KIII β on lysosomes has been investigated in greater detail. The only known interaction partner to date for CaBP7 is PI4KIII β (Mikhaylova et al., 2006) and together with the finding of CaBP7 co-localisation to lysosomes the question emerged whether CaBP7 might also regulate the lysosomal pool of PI4KIII β (Rajamanoharan et al., 2015). Results from this chapter highlight that CaBP7 overexpression does indeed influence lysosome distribution in HeLa cells leading to a clustering phenotype very similar to that observed when PI4KIII β activity is directly perturbed (Figure 3.6.). This is consistent with the published data from Sridhar *et al.* where down-regulation of PI4KIII β or overexpression of PI4KIII β^{D656A} generated the same phenotype. Importantly, this suggests that CaBP7 can directly influence the lysosomal pool of PI4KIII β and hence regulate lysosomal positioning in the cell. Moreover, the regulation of PI4KIII β on lysosomes by CaBP7 is Ca^{2+} independent, which has been evaluated by examining the effect of EF hand mutants of CaBP7 on lysosomal clustering. All CaBP7 EF hand mutations (either single or double mutations) led to lysosome clustering (Figure 3.7.), this is again consistent with CaBP7s interaction with PI4KIII β on the TGN (Burgoyne & Haynes,

2010). Collectively these data indicate that CaBP7 has a novel function in the regulation of lysosomal PI4KIII β .

Even though CaBP7 has shown no localisation on early endosomes (Figure 3.4.), the overexpression of CaBP7 could possibly alter and influence early endosomal sorting pathways. To address this issue the early endosome markers Rab5a and M6PR were tested for their localisation upon CaBP7 overexpression. No deviations in early endosomal morphology or distribution were observed which is consistent with a CaBP7 function specifically on late endosomes and lysosomes (Figure 3.9.).

Consistent with a model where lysosome resident CaBP7 regulates lysosomal PI4KIII β , CaBP7 overexpression significantly depleted cellular PI4P levels (Figure 3.10 A, C), which might account for the lysosomal clustering observed on CaBP7 overexpression. These data together imply that CaBP7, as on the TGN, negatively regulates PI4KIII β on lysosomes. However there are certain restrictions and limitations to these results. Phosphoinositide homeostasis is maintained by the activity of a complex kinase and phosphatase network (T. Balla, 2013; Di Paolo & De Camilli, 2006). The global reduction of PI4P upon PI4KIII β inhibition might be counteracted and compensated for by activation of other PI4Ks (PI4KII α , PI4KII β , PI4KIII α) or Phosphoinositide 5-phosphatase (responsible for conversion of PI (4,5) P₂ \rightarrow PI4P) thereby liberating more PI4P. This explains why the reduction of PI4P in this study is only partial although still statistically significant.

It would have been useful to specifically evaluate PI4P levels only on the lysosomes upon CaBP7 overexpression, to directly connect it to the

lysosome clustering phenotype. However it is problematic to distinguish lysosomal PI4P staining from other pools of this lipid such as those present on the endoplasmic reticulum, PM and Golgi apparatus. Inhibition of Golgi and lysosomal resident PI4KIII β (by CaBP7 overexpression) triggered a measurable depletion of cellular PI4P levels as determined by immunofluorescence microscopy. These data support the importance of this kinase in PI4P production and its possible role in various cellular processes as PI4P is emerging as a major signalling molecule (De Matteis et al., 2013). Additionally Phosphatidylinositol 4,5 bisphosphate (PI (4,5) P₂) levels were also analysed to eliminate the possibility that the reduction of PI4P could significantly affect levels of related phosphoinositides. PI4P is the major precursor of PI (4,5) P₂ generation (Hammond et al., 2012), therefore the reduction of PI4P upon CaBP7 overexpression might conceivably lead to an indirect reduction in PIP₂ levels. Even though a small change in PI (4,5) P₂ levels could be observed on CaBP7 overexpression, there was no substantial reduction of PI (4,5) P₂ levels in CaBP7 expressing cells compared to control cells that could account for the dramatic lysosomal clustering phenotype (Figure 3.10. B, C). The slight reduction of PI (4,5) P₂ in CaBP7 expressing cells is mostly likely due to the reduction of PI4P, however since statistical analysis revealed no significant decrease this should not interfere with PI (4,5) P₂ dependent functions in the cell.

To further support the reduction of PI4P levels upon CaBP7 overexpression dot blot experiments were performed on lipids extracted from larger cell populations. Dot blot analysis from lipids extracted from CaBP7- and control mCherry-N1 overexpressing cells did not show any significant differences

between the two samples (Figure 3.10. D). This might be explained due to limiting transfection efficiency; for example, transient expression of CaBP7 is typically variable in these samples (a mixture of cells expressing low to high levels of transfected protein) and therefore PI4P was not significantly reduced. A further confounding factor is that at best, transient transfections yield 50-70% transfection efficiency and in these assays was never greater than 40%, which might not be sufficient to detectably decrease PI4P levels from large cell populations treated in this way. Technical differences between the two types of PI4P analysis employed could also explain the different results obtained. PI4P positive particle counting by immunofluorescence staining selects for CaBP7 expressing cells only. Dot blot analysis looks at lipid from all cells, both transfected and un-transfected along with cells having varying levels of expressed CaBP7 protein. This assay will therefore be inherently more 'noisy' and difficult to generate a measurable result from. Another approach to validate PI4P reduction upon CaBP7 overexpression more accurately by dot blot analysis would be to either generate stably expressing CaBP7 cell lines from which lipid can be extracted and examined, or transiently overexpressing CaBP7 followed by fluorescence activated cell sorting (FACS) to obtain only mCherry-CaBP7 expressing cells, from which lipid can be extracted and assessed.

Lysosomes are ubiquitous membrane bound intracellular organelles with central roles in degradation and recycling of macromolecules (Luzio et al., 2007). However advances in lysosome research have discovered far more roles for lysosomes suggesting the typical "waste-bag" perception may not be completely accurate. Lysosomes have been shown to have a wide variety

of important cellular functions including PM repair (Luzio et al., 2007; McNeil, 2002; A. Reddy et al., 2001), cholesterol homeostasis (Cheruku, Xu, Dutia, Lobel, & Storch, 2006), cell death (Kirkegaard & Jäättelä, 2009) as well as in various human diseases (Kirkegaard & Jäättelä, 2009; Maxfield, 2014; L. Zhang, Sheng, & Qin, 2009). The discovery of PI4KIII β on lysosomes and the discovery of its regulation by CaBP7 might indicate additional important functions of lysosomes. Organelle functions are intimately linked to their localisation and distribution inside the cell. The correct localisation of lysosomes modulated by PI4KIII β activity, could link localised phosphoinositide signalling and metabolism to dynamic movement of lysosomes, which may underpin so far uncharacterized cellular functions. CaBP7 influences this phenomena and is consistent with its known function as a PI4KIII β regulator however it is also possible that CaBP7 might exert other lysosome specific functions and this warrants further investigation.

In recent years lysosomes have additionally been characterized as Ca²⁺ storage organelles with a luminal Ca²⁺ concentration of ~600 μ M (Appelqvist et al., 2013; Christensen et al., 2002), similar to that measured in the ER (Bygrave & Benedetti, 1996) and mitochondria, which are the two major intracellular Ca²⁺ storage organelles. The association of a Ca²⁺ binding protein, CaBP7, with lysosomes could conceivably influence lysosome specific Ca²⁺ signalling events. Even though the lysosome clustering phenotype triggered by CaBP7 was Ca²⁺ independent, CaBP7 might still be implicated in other Ca²⁺ dependent functions on the lysosomes. More research is required to gain further insights into all potential functions of CaBP7 on lysosomes.

3.4. Summary

Data presented in this chapter confirmed that CaBP7 is endogenously expressed in HeLa cells where it localises to the Golgi, PM and lysosomes. Further investigation into CaBP7s role on lysosomes revealed its regulation of the lipid kinase PI4KIII β . The modulation of the lysosomal pool of PI4KIII β has been shown to be Ca²⁺ independent. The data presented are consistent with CaBP7 inhibiting PI4KIII β activity on the lysosomes, reducing PI4P levels and therefore causing defects in lysosome trafficking. This in turn triggers visible lysosome clustering at the perinuclear region instead of displaying a disperse distribution throughout the cytoplasm. Collectively, the data presented in this chapter deliver new insights into CaBP7 function.

Chapter 4

A functional requirement for CaBP7 during cell division

4.1. Introduction

Mitosis is a fundamental process in complex multicellular organisms, including humans, which is required for normal growth, development, tissue repair and ageing. Defects in mitosis can lead to the state of aneuploidy (abnormal amounts of genetic material), an accepted driver of cellular transformation and cancer (Kops, Weaver, & Cleveland, 2005; Rajagopalan & Lengauer, 2004). Mitosis by definition is the process whereby a single cell duplicates its chromosome number and all its genetic content to be ultimately apportioned into daughter cells (Nigg, 2001). Mitosis represents only a fraction of the cell cycle. The HeLa cell cycle usually lasts around 18-20 hours and the majority of this time is occupied by interphase (16-18 hours), which can be further divided into G1 (Gap1), S (Synthesis) and G2 (Gap2) phases (Diagram 4.1.). G1 can take up to 10 hours and is the preparation phase for DNA synthesis; here the cell functions normally however it will typically increase in size to accommodate impending duplication of its DNA. S phase follows G1 phase, whereby the cell duplicates its chromosome number, this can vary between 4-5 hours. This is then followed by 2-3 hours of G2 phase where the cell continues growing in size and preparing for division (Norbury & Nurse, 1992; Vermeulen et al., 2003). Mitosis follows G2 phase and is the shortest phase of the cell cycle requiring around 1-1.5 hours for completion. As aforementioned, defects in mitosis can lead to cellular transformation and therefore it is a tightly regulated process. Mitosis contains five sub-phases based on chromosomal localisation and state of the mitotic-spindle: Prophase, Prometaphase, Metaphase, Anaphase and Telophase followed by cytokinesis. Each phase of mitosis is subject to its own unique

set of molecular events and regulatory checks. Under normal circumstances this highly ordered process and its associated stringent quality controls ensure successful completion of cell division.

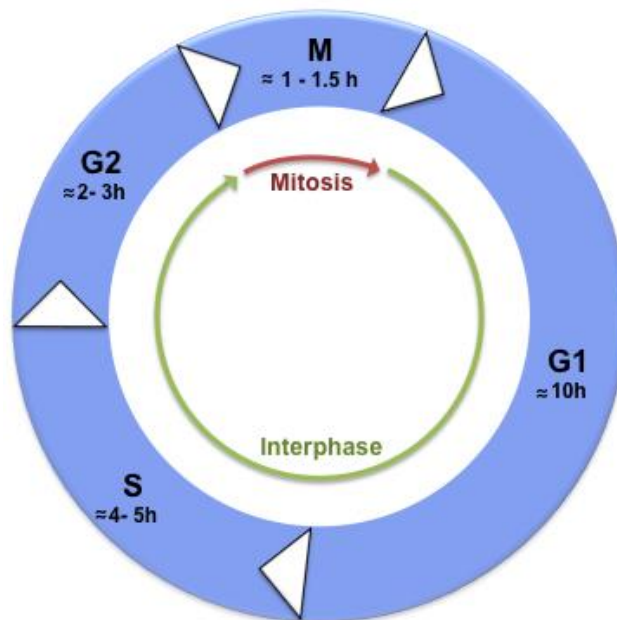


Diagram 4.1. Mammalian cell cycle. Organisation and relative duration of cell cycle phases. Interphase includes G1, S and G2 phases and Mitosis can be subdivided into Prophase, Prometaphase, Metaphase, Anaphase and Telophase followed by cytokinesis.

Various studies dedicated to deciphering the interplay between proteins in mitosis have identified a conserved core proteome critical to this process. These can be categorized into five sub-groups according to their specific participation during cell division: 1) Proteins at the central spindle; 2) RhoA and its regulators and effectors; 3) Non-muscle myosin II; 4) Actin and direct regulators of its assembly into filaments and 5) Factors required for trafficking and fusion of membrane vesicles (Barr & Gruneberg, 2007). Additionally cyclins and cyclin dependent kinases (Cdks) are recognised as master regulatory proteins involved in ensuring proper progression through the cell

cycle. Cyclins undergo dynamic synthesis and degradation during cell division. Once synthesised, cyclins act as activating proteins by binding to Cdks and forming a Cdk-cyclin complex. This complex then signals to the cell to pass to the next cell cycle phase (Malumbres & Barbacid, 2009). These and other proteins monitor key checkpoints to detect any possible defects during DNA synthesis and chromosome segregation. In the incidence of error, cell cycle progression is placed on hold to allow repair and to prevent transmission of faults into daughter cells.

The overall result of these highly coordinated events is the generation of two genetically identical daughter cells. The final phase of mitosis, telophase occurs when the duplicated sets of chromosomes reach their respective spindle poles in readiness for segregation into the new daughter cells. During this period the chromosomes revert back to their normal compact state as during interphase and the nuclear envelope reforms. At this point the mother cell is equipped for physical separation, which finally occurs during the process of cytokinesis. This step can be counted as the 6th stage of mitosis but is often regarded as a distinct phase of the cell cycle. Cytokinesis itself comprises a coordinated set of unique steps, which act to mediate the successful physical separation of daughter cells (Fededa & Gerlich, 2012). Firstly, the plasma membrane (PM) furrows and ingresses at the cell equator, which involves the constriction of an extensive actomyosin II cortical network (Komatsu, Yano, Shibata, Tuft, & Ikebe, 2000; Matsumura, 2005). Thereafter, a fine intercellular bridge connects daughter cells for the duration of cytokinesis, a linkage stabilised by the septin proteins (Estey et al., 2010; Kinoshita et al., 1997). The intercellular bridge is made of dense bundles of

antiparallel microtubules that overlap at the midbody, a structure responsible for intercellular bridge organization (Fededa & Gerlich, 2012). The electron dense midbody ranges between 1-2 μm in diameter whereas the narrowing bridge to the sides is less than 0.5 μm (Green et al., 2012). Finally, abscission occurs when the bridge is severed by the endosomal-sorting complex required for transport (ESCRT) cytoskeleton machinery (Caballe & Martin-Serrano, 2011; Elia, Sougrat, Spurlin, Hurley, & Lippincott-Schwartz, 2011).

In 2010, Neumann *et al.* performed a high-throughput phenotypic RNAi screen in order to identify proteins specifically required during mitosis and cytokinesis (Neumann et al., 2010). Employing computational methods with stringent significance thresholds such as morphology, nuclear mobility and proliferation rate, candidate proteins were identified for their roles at various stages of mitosis. In this study, the cellular depletion of CaBP7 elicited cytokinesis failure (Neumann et al., 2010). CaBP7 depletion caused failure in daughter cell separation, leading to coalescence into a single cell that was binucleate. Polynucleate cells were also observed in these analyses indicating that CaBP7 loss of activity did not affect the majority of cell cycle related events but that cytokinesis was specifically inhibited at each attempt at cell division. CaBP7 was the only Ca^{2+} binding protein identified in this screen, although a role for calmodulin (CaM) in cell cycle progression has been known for many years. CaM overexpression and down regulation studies showed that CaM levels control the rate of cell cycle progression (Rasmussen & Means, 1989). Furthermore CaM has been demonstrated to have various roles throughout cell cycle progression including stimulating

expression of genes leading to activation of Cdks, activating enzymes required for DNA synthesis, inducing nuclear envelope break down and specific effects on cytokinesis (N. Takuwa, Zhou, & Takuwa, 1995; Y. Y. Yu, Dai, Pan, Chen, & Li, 2005). Ca^{2+} signals have been implicated as important during mitosis in some model cell systems, particularly those amenable to imaging and manipulation such as sea urchin embryos and *Xenopus* eggs (Whitaker, 1997; Whitaker & Patel, 1990). However the role of Ca^{2+} during somatic cell division in mammalian cells is highly controversial. During sea urchin egg fertilization (Steinhardt, Zucker, & Schatten, 1977) transient Ca^{2+} signals have been reproducibly measured. Further on during mitosis entry and exit, small Ca^{2+} signals could be measured in the early embryonic sea urchin (Whitaker & Patel, 1990). Similar results were obtained in studies of *Xenopus* egg fertilization with Ca^{2+} sensitive microelectrodes (Busa & Nuccitelli, 1985; Whitaker, 1997; Whitaker & Patel, 1990) and in mouse oocytes (Cuthbertson, Whittingham, & Cobbold, 1981). Additional proof was delivered by blocking Ca^{2+} signalling with Ca^{2+} selective chelators such as BAPTA, which prevented fertilization and blocked mitosis in *Xenopus* and sea urchin eggs (T. Noguchi & Mabuchi, 2002; Steinhardt et al., 1977; Webb, Li, & Miller, 2008) suggesting an important role of Ca^{2+} signalling during mitosis. This is not surprising as Ca^{2+} is a universal intracellular messenger that has been implicated in almost all cellular functions. Ca^{2+} signalling has also been observed specifically during cytokinesis, however its exact mechanism of action and function is yet to be studied (R. Wong et al., 2005). In *Danio rerio* (zebrafish) embryos, increased Ca^{2+} signalling could be measured along the equator of the cleavage furrow with an additional Ca^{2+}

elevation during cleavage completion (Webb et al., 2008; Whitaker & Patel, 1990). In this study Ca^{2+} chelators were also employed, which led to cytokinesis failure. Inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs) and endoplasmic reticulum resident proteins co-localise to either side of the ingression cleavage furrow in mouse oocytes and zebrafish embryos (Cuthbertson et al., 1981; K. W. Lee, Webb, & Miller, 2003), and influencing IP_3Rs , or IP_3 levels directly, results in cytokinesis defects, showing that the origin of Ca^{2+} signalling during cytokinesis is, at least in part, from the ER Ca^{2+} store (K. W. Lee et al., 2003; W. M. Li, Webb, Chan, & Miller, 2008).

Additionally, phosphoinositide metabolism has been reported to play important roles during mitosis (Brill et al., 2011; Echard, 2012; Janetopoulos & Devreotes, 2006) and specific functions for $\text{PI4KIII}\beta$ have been identified from studies in model organisms. Cellular depletion of the $\text{PI4KIII}\beta$ homologues in yeast (PIK1) (J.-S. Park, Steinbach, Desautels, & Hemmingsen, 2009), in the trypanosome *T. brucei* ($\text{TbPI4KIII}\beta$) (Rodgers et al., 2007) and in *Drosophila melanogaster* (Four wheel drive (Fwd)) (Polevoy et al., 2009) led to cytokinesis failure, underlining an essential requirement for this protein for successful cytokinesis completion. Interestingly, Polevoy *et al.* showed the partial rescue of the cytokinesis defect of *fwd* mutant flies upon overexpression of a catalytically inactive version of Fwd protein. These results suggest catalytic as well as non-catalytic roles for $\text{PI4KIII}\beta$ during cytokinesis. In accordance with this result overexpression of a constitutively active form of the small GTPase Rab11 could also partially rescue the phenotype (Polevoy et al., 2009). Since Rab11 is recruited by $\text{PI4KIII}\beta$ during cytokinesis, this also suggested a non-catalytic function of $\text{PI4KIII}\beta$ during

cytokinesis. Furthermore, PI4P, the product of PI4KIII β , has also been implicated in mitosis (Polevoy et al., 2009). It is likely, that both an excess and underproduction of PI4P could cause mitosis failure and therefore the stringent synthesis and removal of this lipid is might be crucial for normal mitosis completion.

The objectives of this chapter were to further investigate the role of CaBP7 and its functions in cytokinesis and how these might possibly connect to PI4KIII β regulation.

4.2. Results

4.2.1. CaBP7 down regulation causes cytokinesis failure

CaBP7 has been identified as an essential protein required for successful cytokinesis in mammalian cells (Neumann et al., 2010). Loss of CaBP7 activity leads to cytokinesis failure and accumulation of cells harbouring >1 nuclei. To verify this observation HeLa cells were transfected with a shRNAi expression plasmid targeted against CaBP7 or a scrambled shRNA control plasmid. 72 hours post treatment cells were fixed and stained with DAPI to visualize nuclear material in order to determine whether an increased frequency of binucleate cells could be observed. Figure 4.1. A and B show representative images of control (scrambled shRNAi) and CaBP7 knockdown (KD, CaBP7 shRNAi) transfected HeLa cells. Expression of a scrambled control CaBP7 shRNAi plasmid generated an 8.4% abnormal nuclei frequency (ANF, the percentage of cells with >1 nuclei), which increased to 22.9% on expression of CaBP7 specific shRNAi (Figure 4.1 D, Table 4.1.).

This 2.7 fold difference in ANF is consistent with the report of Neumann *et al.* Importantly, the cytokinesis defect generated by CaBP7 protein depletion could be specifically rescued by co-expression of a shRNAi resistant version of the protein. The rescue experiments generated an 11.4% ANF, close to the value of 8.4% obtained with control shRNAi plasmid transfection (Figure 4.1. C and D, Table 4.1.). Silencing was verified by Western blot examination of the influence of the various shRNAi plasmids on exogenously expressed mCherry-CaBP7 protein. Anti-RFP antibody was used to detect the mCherry protein and illustrated specific depletion of mCherry-CaBP7 by the CaBP7 specific shRNAi, resilience of the silencing resistant mCherry-CaBP7 construct to the same shRNAi and failure of the scrambled control shRNAi to influence mCherry-CaBP7 levels (Figure 4.1. E). The same blot was re-probed for actin as a loading control, which highlighted no difference in protein content between the samples.

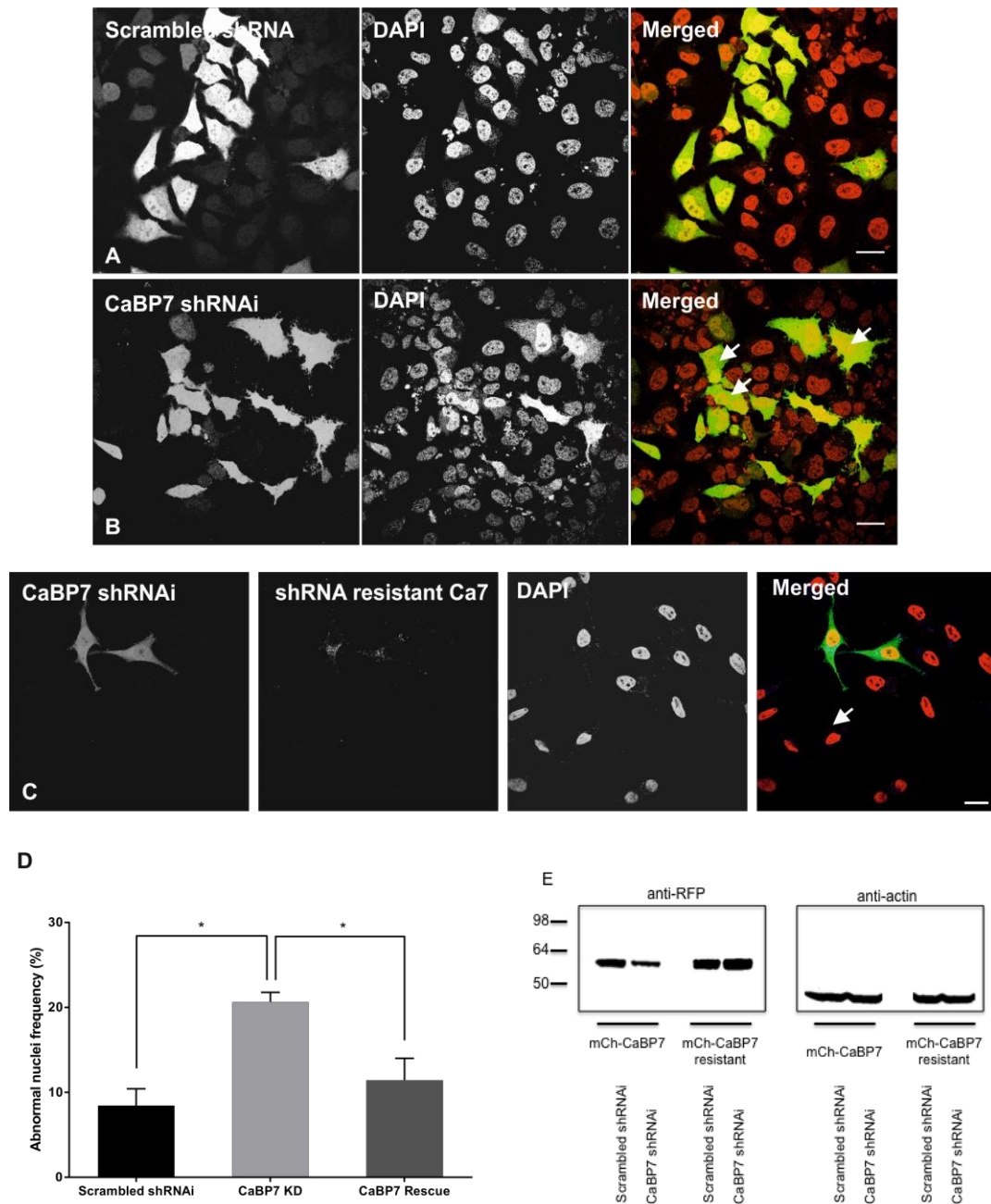


Figure 4.1. CaBP7 depletion in HeLa cells. Representative confocal images of CaBP7 shRNAi study, quantification and western blot. A) HeLa cells expressing scrambled shRNAi (green), DAPI (red), B) HeLa cells expressing CaBP7 shRNAi (green) and DAPI (red), C) HeLa cells expressing CaBP7 shRNAi (green), shRNAi resistant CaBP7 (blue) and DAPI (red). D) Quantification of abnormal cells from CaBP7 knockdown and rescue experiment, all data is plotted as mean \pm SEM. One-way ANOVA with multiple comparisons was performed (* $p < 0.05$). E) Western blot of exogenously expressed mCherry-CaBP7 and shRNAi resistant form of mCherry-CaBP7 (52 kDa) upon CaBP7 depletion (anti- RFP) and loading control actin (42 kDa).

4.2.2. PI4KIII β over activation inhibits cytokinesis

PI4KIII β has a documented involvement in cell division (Polevoy et al., 2009; Rodgers et al., 2007) and is the only known interaction partner for CaBP7 (Burgoyne & Haynes, 2010; Mikhaylova et al., 2009), which is a negative regulator of PI4KIII β . Hence the knockdown of CaBP7 might be predicted to allow PI4KIII β to be basally active, and so the question arose as to whether CaBP7 depletion could elicit a cytokinesis defect due to loss of PI4KIII β repression. In the presence of activating proteins including Arf1 and NCS-1 there also exists the possibility that PI4KIII β activity could be stimulated above basal levels when CaBP7 is absent. Therefore these studies were extended to examine the influence of overexpression of PI4KIII β and its activators on cytokinesis to determine if increased PI4KIII β activity, and increased PI4P production could explain the effect of CaBP7 depletion on cytokinesis arrest. As elaborated on above, PI4P has emerged as an important signalling lipid and has been shown to play a role in mitosis (De Matteis et al., 2013; Tan & Brill, 2014).

Figure 4.2. A-D display representative images of HeLa cells overexpressing various constructs stained with the nuclei marker DAPI. For overexpression studies EYFP was employed as a control protein, which caused an 8.2% ANF (Figure 4.4., Table 3.1.) similar to control shRNAi expression (Figure 4.1. D). Overexpression of PI4KIII β and its activators NCS-1 and Arf1 that would be predicted to increase PI4P production by PI4KIII β , generated ANFs of 17.9%, 19.6% and 13.5% respectively (Figure 4.2. B-D, 4.4.). NCS-1 and Arf1 have been shown previously to stimulate PI4KIII β activity (Haynes et al., 2005; Zhao et al., 2001). These results suggest that the effect of CaBP7

knock down on cytokinesis defect might be due to increased PI4KIII β activity and amplified PI4P production.

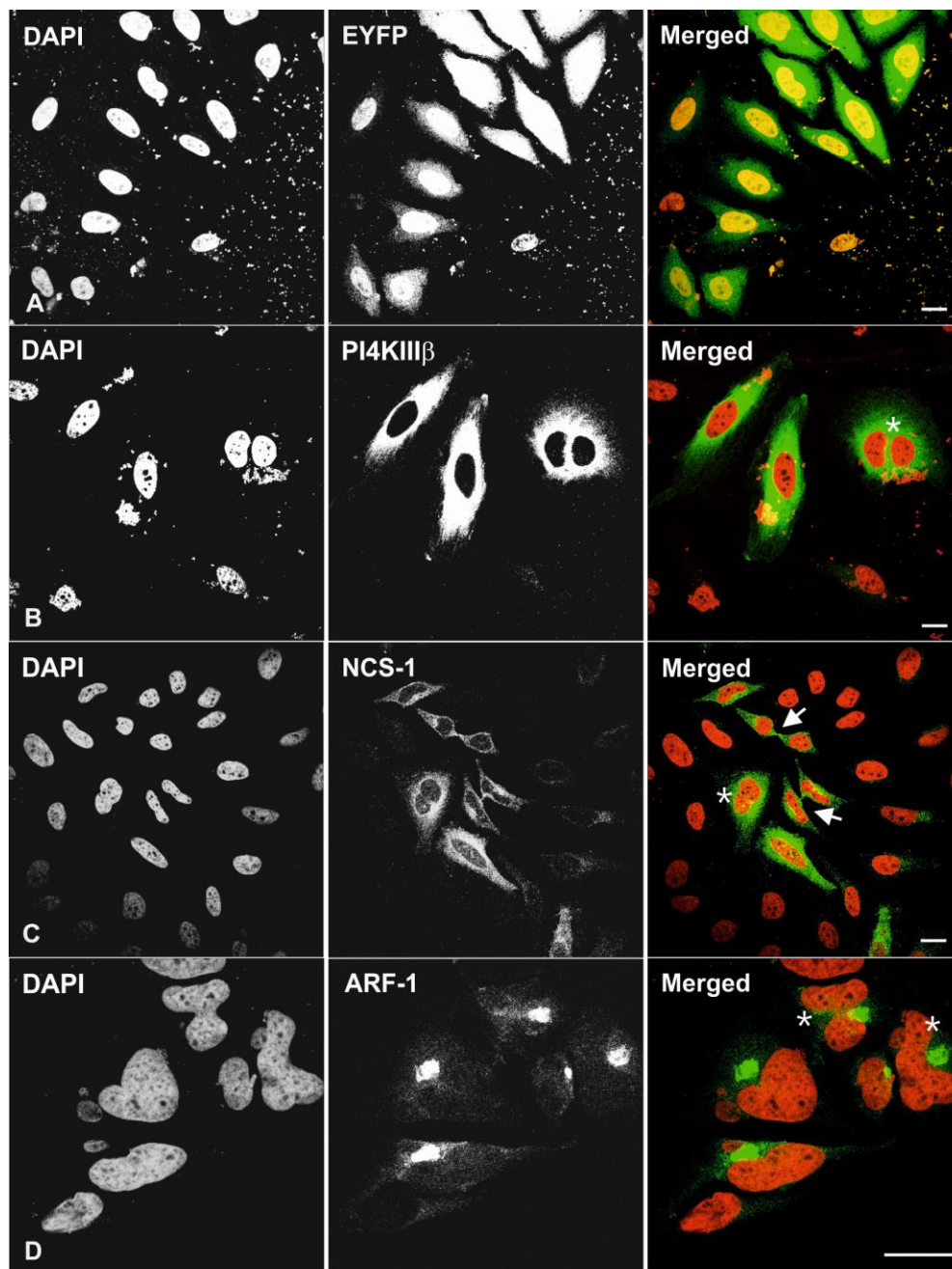


Figure 4.2. Activation of PI4KIII β and its influence on ANF. Example confocal images of HeLa cells expressing PI4KIII β and activators and stained with DAPI (red). All overexpressed constructs are green in the overlay images. HeLa cells expressing: A) EYFP (control); B) PI4KIII β ; C) NCS-1 and D) ARF1. Single cells harbouring >1 nuclei are highlighted with an asterisk and cells at cytokinesis with an arrowhead. Scale bars = 25 μ m.

In contrast, overexpression of either mutant PI4KIII β ^{D656A} (the catalytically inactive kinase) or wild type CaBP7, both of which would be predicted to antagonise PI4P production by endogenous PI4KIII β , generated ANFs similar to those observed with control EYFP expression (7.8% and 6.8% ANF respectively) (Figure 4.3. A-C, 4.4, Table 4.1.). This further implies that only the catalytically active form of PI4KIII β is detrimental to cytokinesis

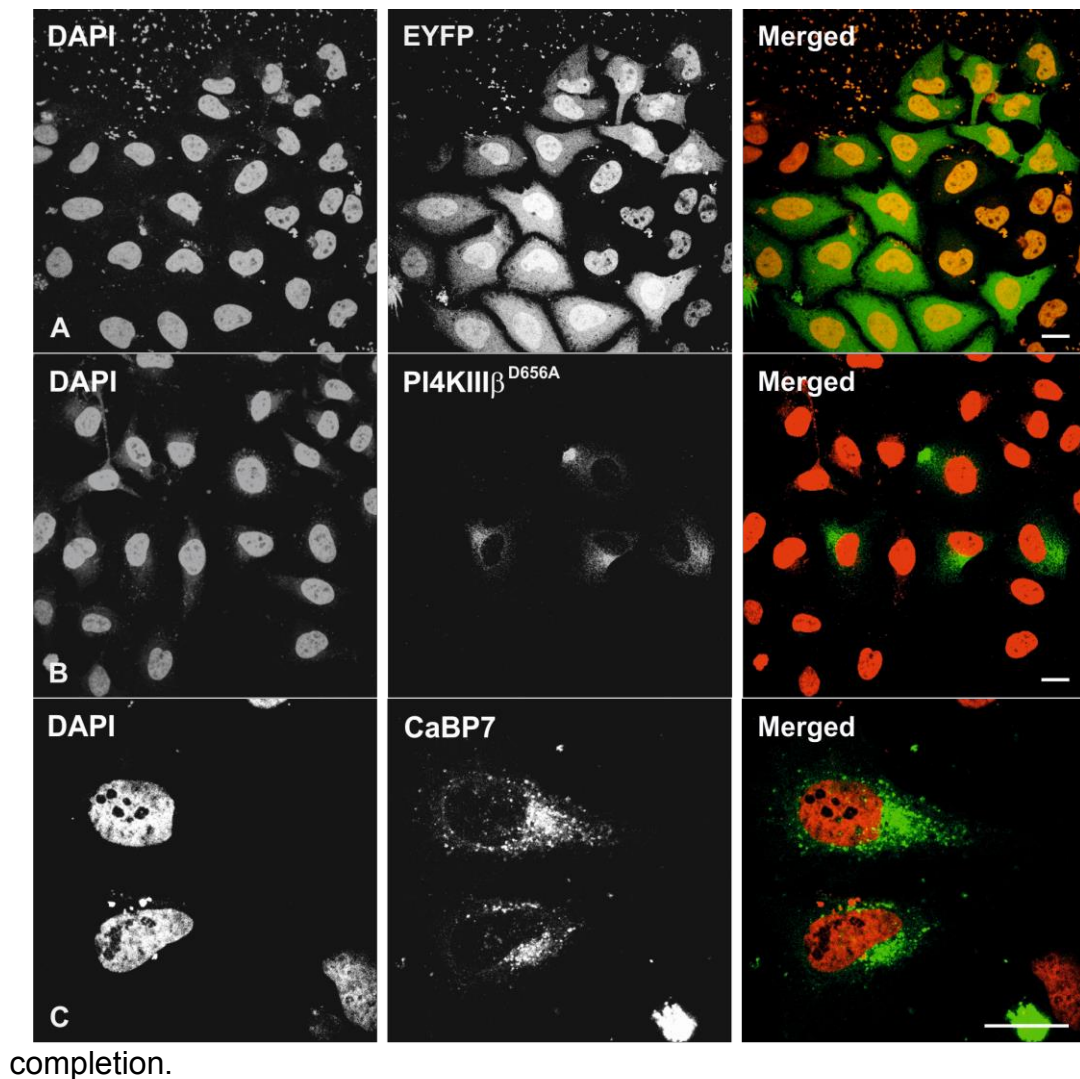


Figure 4.3. PI4KIII β inhibition and its influence on ANF. HeLa cells expressing various constructs (green) were fixed and stained with DAPI (red). A) EYFP (control); B) PI4KIII β ^{D656A} and C) CaBP7. Scale bars = 25 μ m.

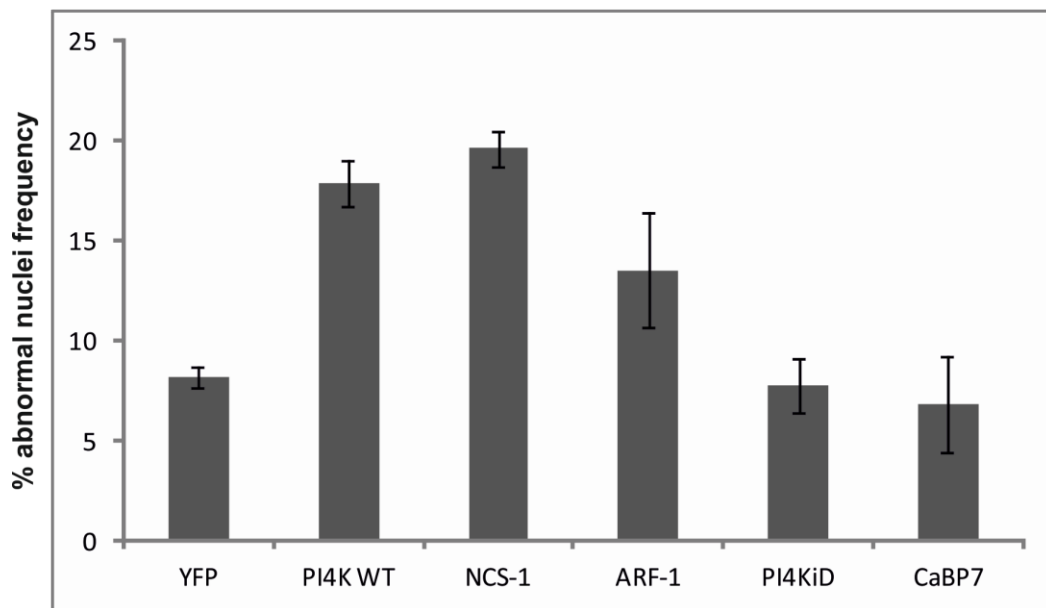


Figure 4.4. Quantification of abnormal cells upon expression of various constructs. One-way ANOVA comparing each data set to EYFP (control) was performed (** $p < 0.01$).

Constructs	Abnormal Nuclei Frequency (%)	\pm SEM	Fold reduction compared to CaBP7 shRNAi	Cells analysed	Number of independent experiments
Scrambled shRNA	8.4	2.0	2.7	1269	3
CaBP7 shRNAi	22.9	0.6	1.0	1108	3
CaBP7 rescue	11.4	2.6	2.0	262	3
EYFP	8.2	0.5	2.8	562	3
PI4KIII β	17.9	1.2	1.3	785	3
NCS1	19.6	1.5	1.2	353	3
ARF1	13.5	2.8	1.7	288	3
PI4KIII β D656A	7.8	1.3	2.9	896	3
CaBP7	6.8	2.4	3.4	350	3

Table 4.1.: Numerical and statistical data for ANF cell assays.

4.2.3. Cell cycle dependent localisation of CaBP7

It is well known that protein functions are highly correlated with their correct localisation hence determination of a protein's precise localisation can reveal information about its potential function(s). During mitosis where extensive membrane trafficking and restructuring occurs the positioning of a specific protein might therefore reveal novel functional insights. In order to further understand CaBP7 involvement in cytokinesis its localisation was tracked during cell division. HeLa cells were immunostained for CaBP7 and α -tubulin along with the nuclear stain DAPI to evaluate CaBP7 positioning throughout cell division. Figure 4.5. shows confocal images of HeLa cells at defined stages of cell division.

At interphase (as shown in Chapter 3 and here Figure 4.5A) CaBP7 could be found both on vesicles distributed throughout the cytosol in addition to a concentration at the perinuclear region, most likely the TGN. In Metaphase cells CaBP7 decorated the periphery of the now rounded cell, and was completely absent from the mitotic spindle (Figure 4.5. B). In early anaphase CaBP7 remained at the periphery of the cell, however some CaBP7 positive vesicles could be seen to localise at the spindle (Figure 4.5. C). Later in anaphase CaBP7 moved further into the spindle region and partially re-distributed between the two nuclei (Figure 4.5. D). In early telophase CaBP7 completely re-localised and displayed a tight and distinctive clustering to either side of the cell nuclei (Figure 4.5. E). At late telophase CaBP7 retained this distinct localisation flanking the intercellular bridge and at the poles of the spindles (Figure 4.5. F).

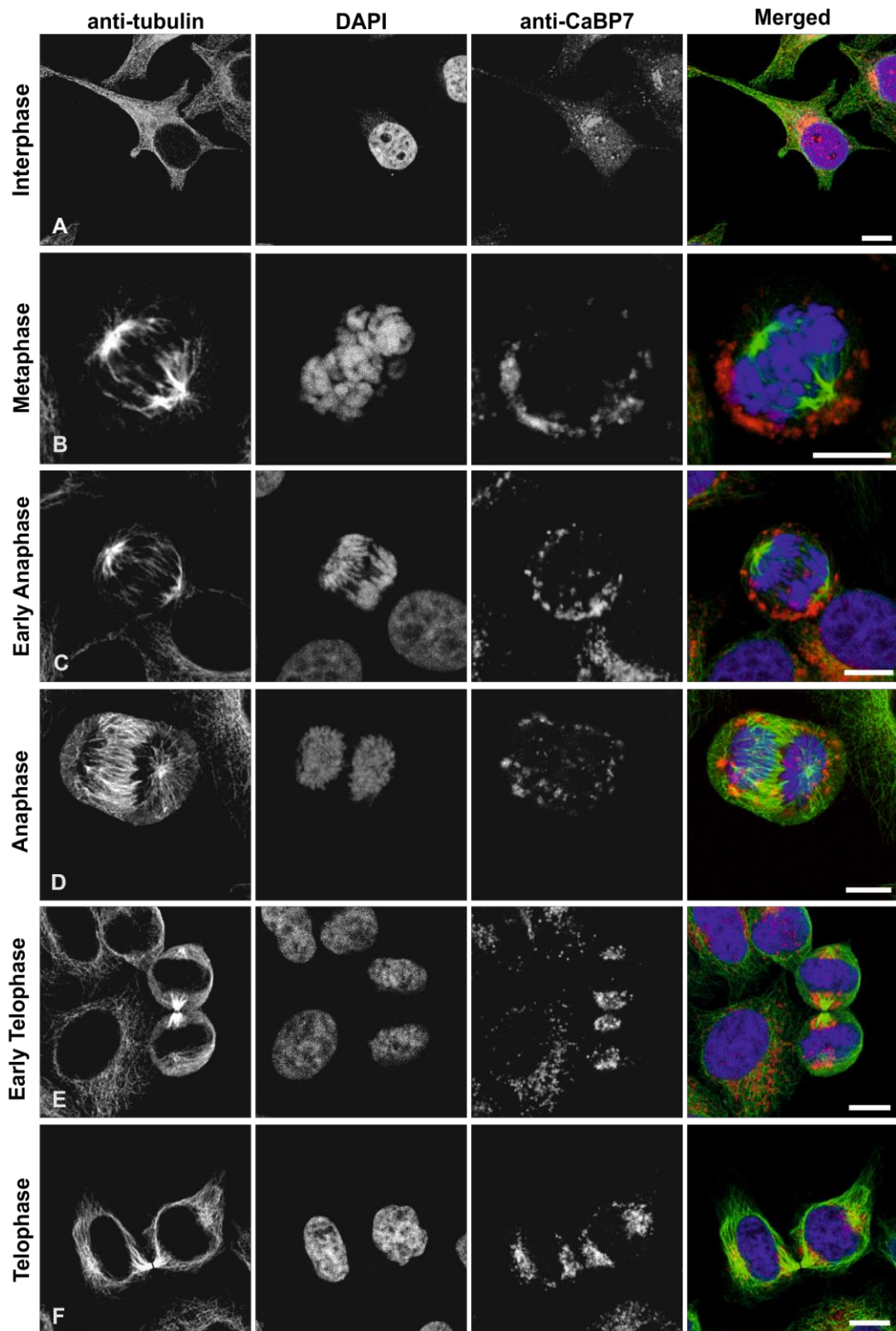


Figure 4.5. CaBP7 localisation during cell division. HeLa cells immunostained with CaBP7 (red), tubulin (green) and DAPI (blue) at various cell cycle stages as indicated on the left (A – F). Areas of co-localisation between tubulin and CaBP7 appear yellow in overlay images. Scale bars = 10 μ m.

4.2.4. Co-localisation of CaBP7 and lysosomes during mitosis

CaBP7 is known to localise to the TGN and to lysosomes (the latter as characterised in Chapter 3 of this thesis,). Observing the distinctive re-distribution of CaBP7 during cell division (Figure 4.5.) led to an examination as to whether this was due to the cell cycle dependent re-distribution of organelles known to be positive for CaBP7 including the TGN and lysosomes. HeLa cells were fixed and stained for the lysosomal marker LAMP1 and for CaBP7 during mitosis to evaluate the extent of co-localisation of these proteins. At interphase CaBP7 and LAMP1 displayed partial co-localisation (Figure 4.6. A), which increased in Metaphase (Figure 4.6. B) and was maximal at early telophase (4.6. C). At late telophase the co-localisation of CaBP7 and LAMP1 declined and appeared similar to the pattern observed at interphase (Figure 4.6. D). The calculated Pearson's correlation coefficients for these analyses mirror the described observations (Table 4.2.). In addition, cells exogenously expressing LAMP1 and CaBP7 constructs were tracked during cell division by live- cell confocal imaging. The still images from a representative video highlight a similar co-localisation of CaBP7 and LAMP1 throughout the cell cycle (Figure 4.7). To confirm these results, a second lysosome marker, CD63, was utilised. HeLa cells stained with CD63, a lysosomal integral membrane protein, and CaBP7 also demonstrated convincing co- localisation between these proteins during mitotic progression (Figure 4.8.) with an increased co-localisation at early telophase as observed for LAMP1 and CaBP7 (Figure 4.8. C, Table 4.2.).

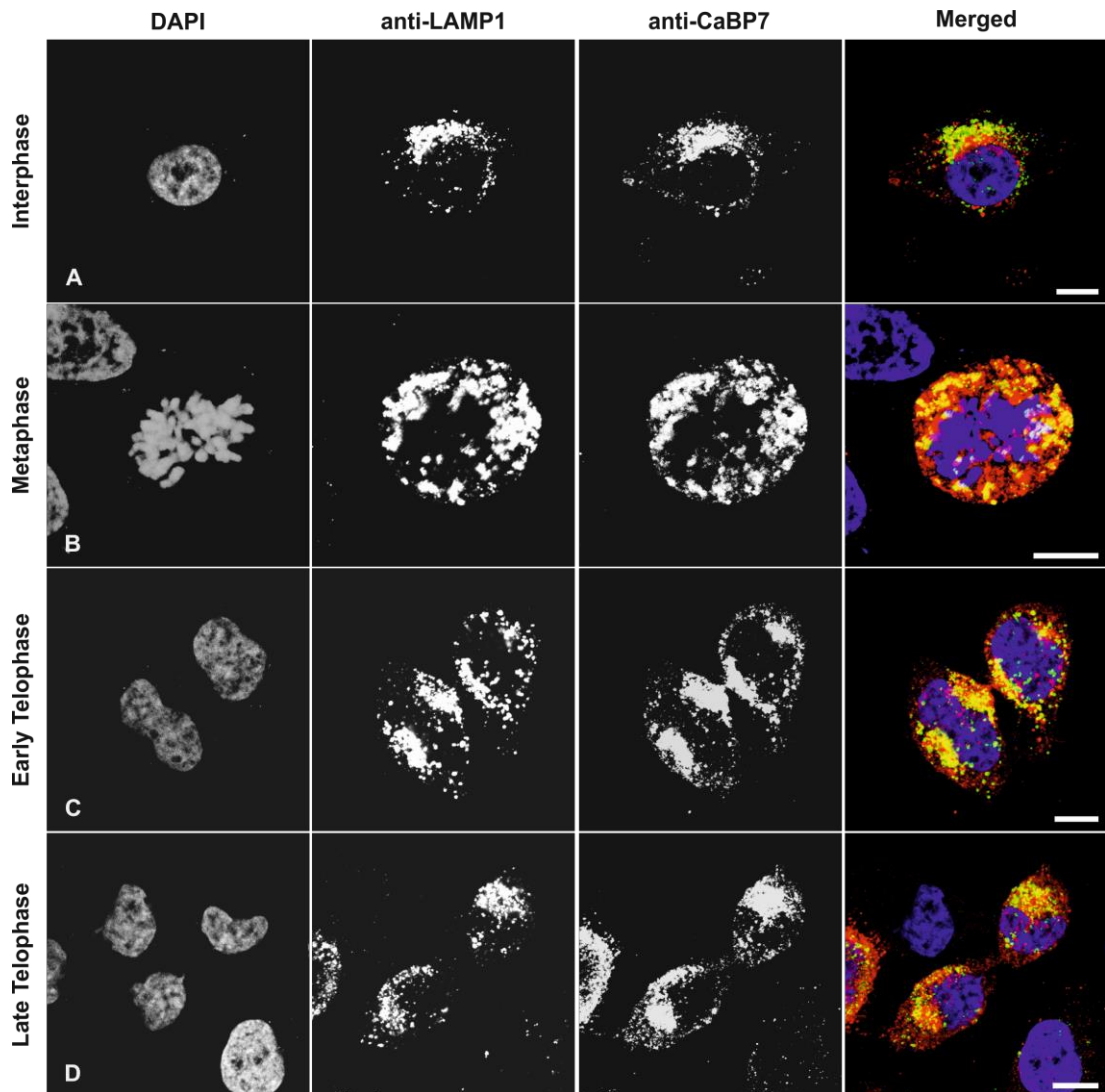


Figure 4.6. Co-localisation of endogenous CaBP7 and Lysosome marker LAMP1 during Mitosis. HeLa cells immunostained for CaBP7 (red), LAMP1 (green) and DAPI (blue) at various stages of cell division as indicated on the left (A- D). Regions of co-localisation appear yellow in the overlay images. Scale bars = 10 μ m.

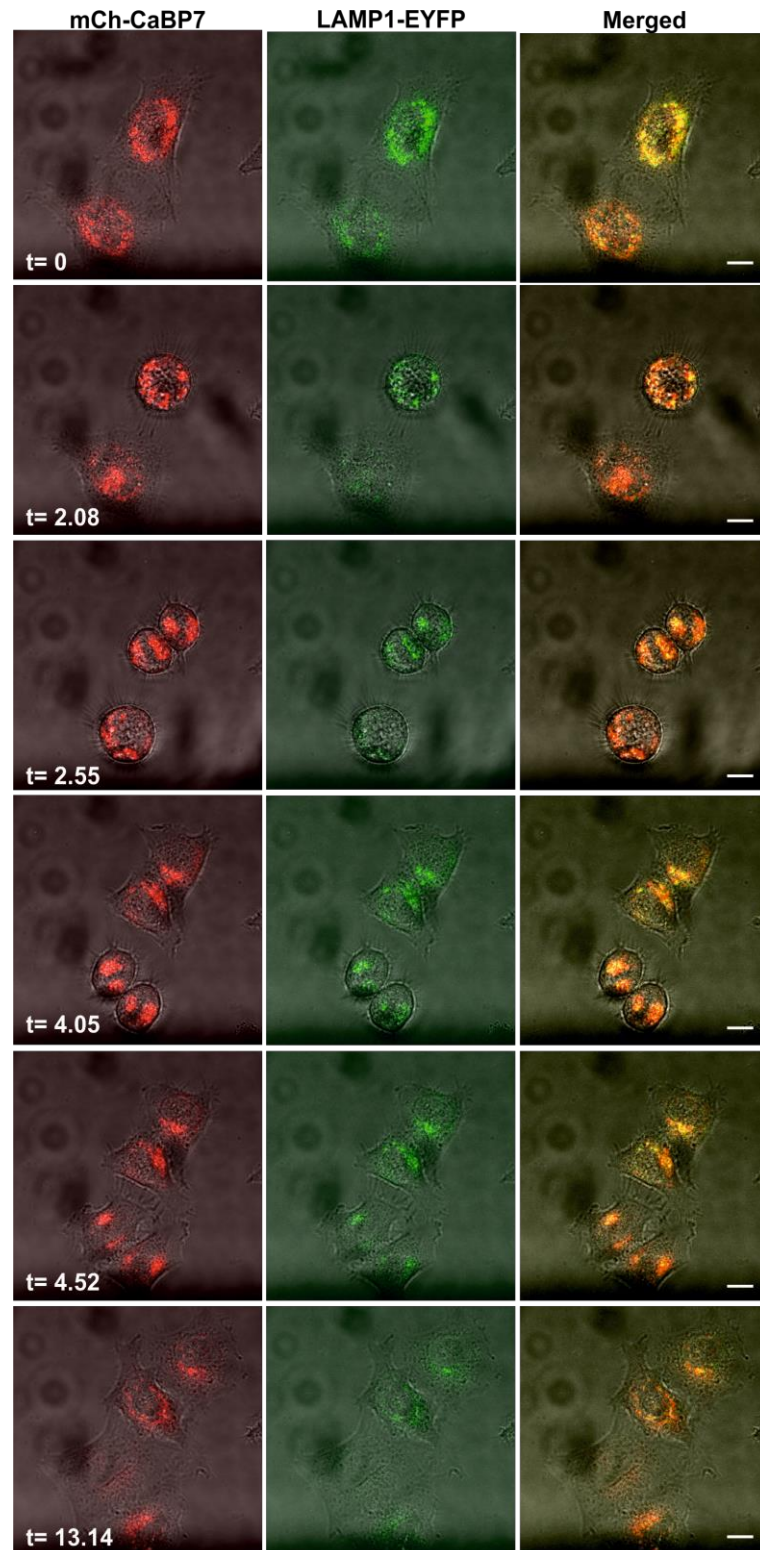


Figure 4.7. Co-localisation of exogenous CaBP7 and LAMP1 during mitosis. HeLa cells expressing mCherry-CaBP7 and LAMP1-YFP were imaged over time during cell division. Still images from a representative recording are depicted. Regions of co-localisation appear yellow in the overlay images. Imaging rate was 1 Frame/11.67 minutes, t= time in hours. Scale bars = 10 μ m.

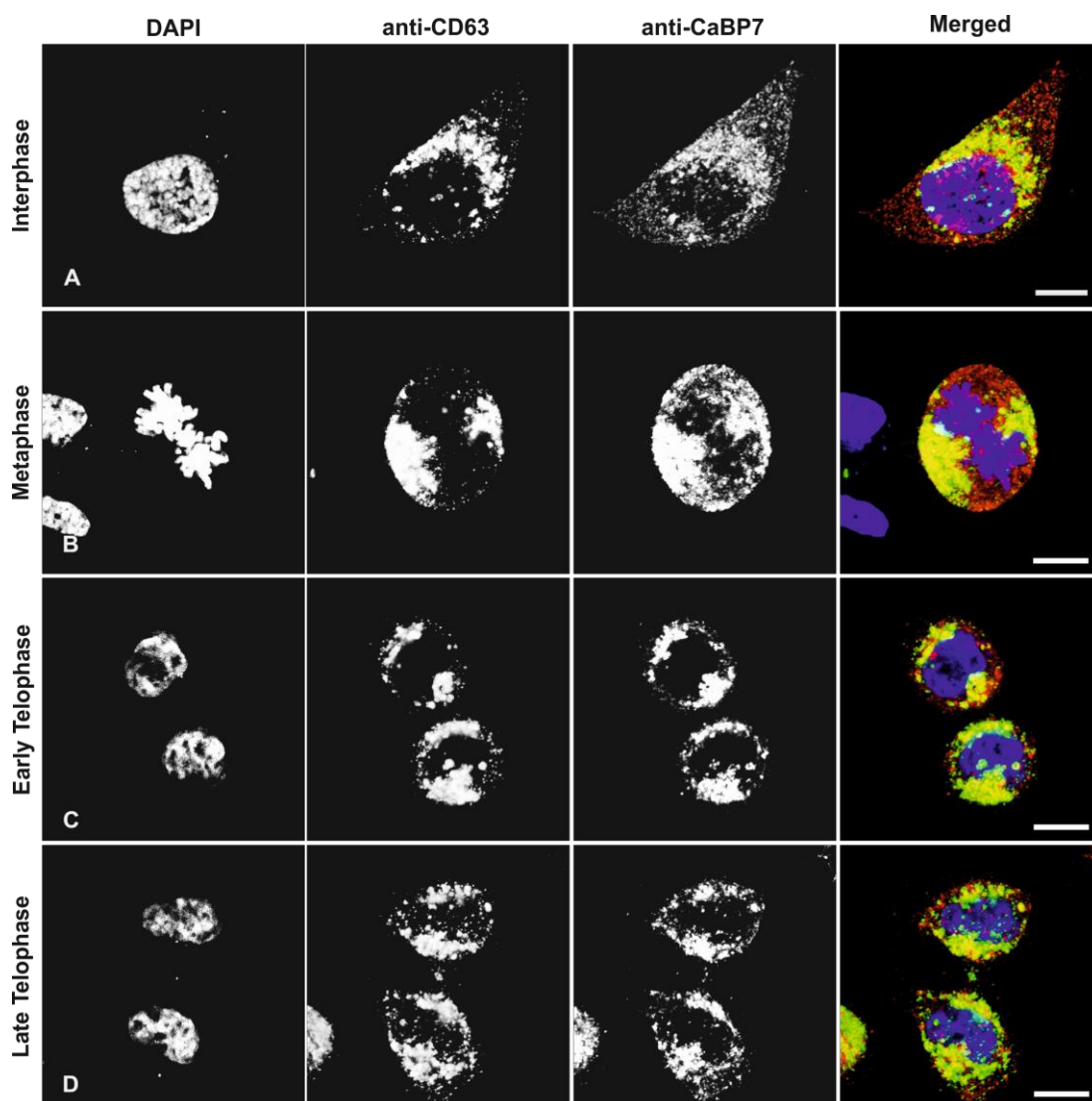


Figure 4.8. Co-localisation of endogenous CaBP7 and lysosome marker CD63. HeLa cells immunostained for CaBP7 (red), CD63 (green), and DAPI (blue) at various stages of mitosis as indicated on the left (A-D). Regions of co-localisation appear yellow in the overlay images. Scale bars = 10 μ m.

To examine the distribution of CaBP7 with the TGN during the cell cycle HeLa cells were fixed and stained for the TGN specific marker, p230, and CaBP7. CaBP7 co-localised at interphase with p230, however at metaphase when the Golgi fragmented, no immunostaining could be detected for p230 (Figure 4.9. B). The Golgi is known to fragment during mitosis, particularly in

metaphase and reforms afterwards (Jesch, Mehta, Velliste, Murphy, & Linstedt, 2001; Shima, Haldar, Pepperkok, Watson, & Warren, 1997; Thyberg & Moskalewski, 1992). Since CaBP7 did not become cytosolic upon Golgi dissipation, and remained associated with vesicles, this strongly suggests, in accordance with the previous findings, that CaBP7 is present on lysosomes and that these organelles themselves dynamically redistribute during mitosis/cytokinesis. Upon Golgi restoration in early telophase p230 protein was again detectable and a partial co-localisation with CaBP7 was apparent that increased in late telophase (Figure 4.9. D). CaBP7's extent of co-localisation through the cell cycle with LAMP1 and p230 is summarized in Table 4.2., emphasizing that CaBP7's maximum co-localisation to lysosomes occurred during mitosis, peaking at early telophase.

Collectively, these results suggest CaBP7 to be principally associated with lysosomes once the Golgi fragments during metaphase and that CaBP7 does not detectably re-distribute to any other organelles. Furthermore these results implicate not only CaBP7 to be important during mitosis but that lysosomes may also have a specific function during this process.

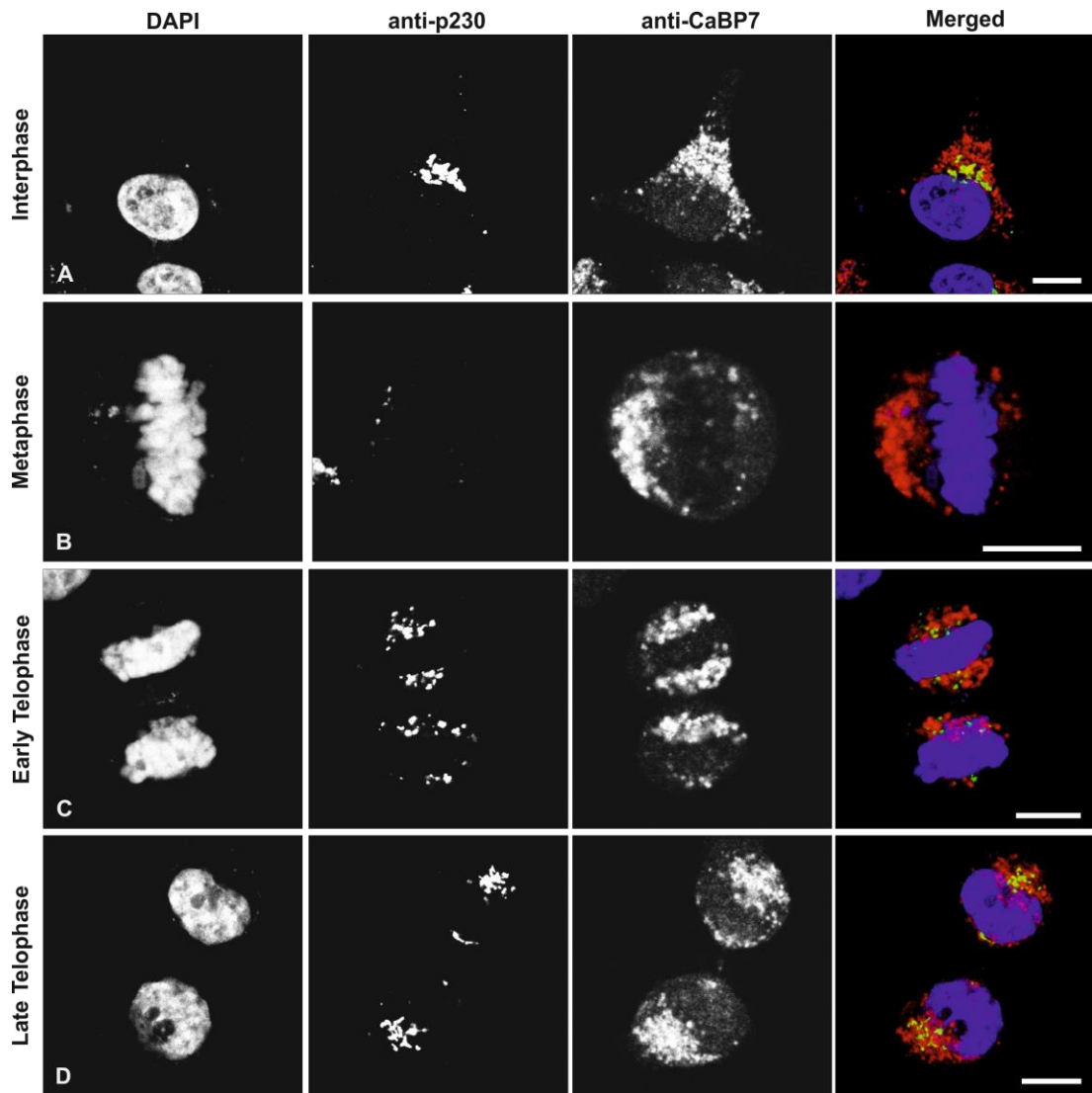


Figure 4.9. Co-localisation of endogenous CaBP7 and Trans- Golgi marker p230. HeLa cells immunostained for CaBP7 (red), Trans-Golgi marker p230 (green) and DAPI (blue) during various cell division stages as indicated on the left (A-D). Regions of co-localisation appear yellow in the overlay images. Scale bars = 10 μ m.

4.2.5. PI4KIII β and lysosomes during mitosis

Previous results have demonstrated CaBP7 co-localisation with lysosomes during mitotic progression, with a distinctive clustering near the intercellular bridge and pole regions at telophase. In fact, based on the study of Neumann *et al.* the role of CaBP7 during mitosis is likely to be at the later stages therefore this distinctive clustering at telophase might be of particular functional relevance. The role of lysosomes during cell division has not been studied and their potential function remains unknown. Redistribution of lysosomes to the intercellular bridge regions during telophase was documented over 25 years ago, however the functional importance of this phenomenon was not investigated (MATTEONI & KREIS, 1987). Interestingly a biochemically distinct pool of PI4KIII β on lysosomes has been discovered and shown in this thesis to be regulated by CaBP7 (Sridhar et al., 2013). Additionally, misregulation of PI4KIII β activity, as characterized in this chapter, influences cell division and CaBP7 positive lysosomes exhibit a unique re-localisation at cytokinesis. A requirement for PI4KIII β during mitosis has been confirmed in various model organisms (Polevoy et al., 2009; Rodgers et al., 2007). Collectively these observations are consistent with a hypothesis where lysosomal PI4KIII β is required for normal mitotic progression. To address this, the co-localisation of PI4KIII β with LAMP1 during mitosis was first examined (Figure 4.10.). PI4KIII β displayed a similar dynamic re-distribution profile to CaBP7. In detail, PI4KIII β exhibited a perinuclear accumulation (consistent with the TGN) at interphase and had limited co-localisation with LAMP1 (Figure 4.10. A). During metaphase and progression through telophase, LAMP1 and PI4KIII β displayed an increasing

degree of co-localisation (Figure 4.10. C, D, Table 4.2.). These data confirm that lysosomal PI4KIII β is present on lysosomes that redistribute during mitosis and cytokinesis

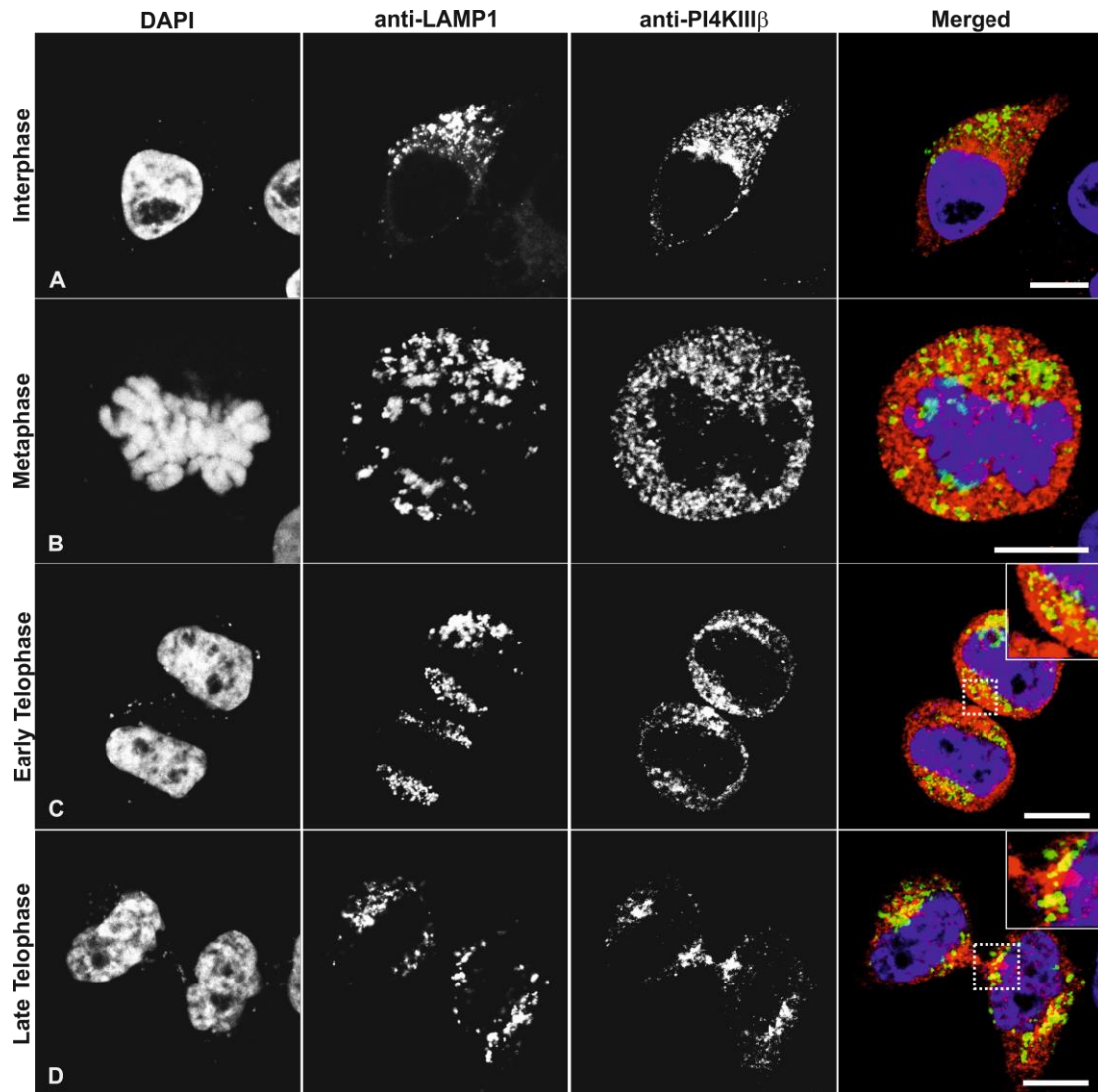


Figure 4.10. Co-localisation of endogenous PI4KIII β and lysosome marker LAMP1. HeLa cells stained for PI4KIII β (red), lysosomes (LAMP1- green) and DAPI (blue) during cell division stages as indicated on the left (A-D). Regions of co-localisation appear yellow in the overlay images. In early and late telophase enlargements of the regions bounded by the white dotted boxes are shown on the top right corner of each image to permit more accurate assessment of co-localisation. Scale bars = 10 μ m.

Mitotic Stages	anti- CaBP7 + anti- LAMP1	anti- CaBP7 + anti-p230	anti- LAMP1 + anti-PI4KIIIβ
<i>Interphase</i>	0.63	0.37	0.51
<i>Metaphase</i>	0.68	0.20	0.48
<i>Early Telophase</i>	0.72	0.28	0.55
<i>Late Telophase</i>	0.59	0.42	0.46

Table 4.2. Pearson's correlation coefficients for co-localisation. Analysis for data from Figure 4.6.; 4.9.; and 4.10. ImageJ plug-in JACoP was utilised to analyse the fluorescence distribution in the immunofluorescence images.

4.2.6. CaBP7 influences PI4P levels during cell division

Previous results in this thesis show that CaBP7, lysosomes and PI4KIII β undergo a marked re-distribution during cell division. These findings, linked to a recent study showing that PI4KIII β resides on lysosomes (Sridhar et al., 2013), and data showing that CaBP7 is a negative regulator of the kinase might suggest a role for CaBP7 regulation of this kinase on lysosomes during cytokinesis (Rajamanoharan et al., 2015). This regulatory model would be of importance given the nature of the cytokinesis failure in CaBP7 depleted cells (Neumann et al., 2010; Rajamanoharan et al., 2015). In chapter 3 the modulation of PI4KIII β by CaBP7 was studied and results showed that the overexpression of CaBP7 inhibits PI4KIII β activity and in turn PI4P levels were decreased. In this part of the study the effect of PI4KIII β inhibition and subsequent depletion of PI4P upon CaBP7 overexpression was investigated during cell division to identify whether levels of this lipid could be correlated with the observed effects of CaBP7. For this purpose HeLa cells expressing exogenous CaBP7 were stained with PI4P antibody and DAPI (to determine

mitotic progression) to observe whether the reduction of PI4P upon CaBP7 expression is limited to interphase or whether it also occurs during the different stages of cell division. Control (un-transfected) cells stained for PI4P during mitosis illustrates PI4P to be present at all stages of mitotic progression at a visibly detectable level (Figure 4.11. A- D). Cells expressing CaBP7 stained for PI4P indicate reduced levels of PI4P at all mitotic stages compared to control (Figure 4.12. A- D), indicating CaBP7 regulates PI4KIII β activity throughout cell division.

To exclude the possibility of CaBP7 influencing PI (4,5) P₂ levels, as PI4P is the major precursor for PI (4,5) P₂, PI (4,5) P₂ levels were also examined during mitosis in un-transfected and CaBP7 overexpressing cells. No discernible changes in PI (4,5) P₂ levels could be observed between control cells (Figure 4.13.) and CaBP7 expressing cells (Figure 4.14.) during mitosis.

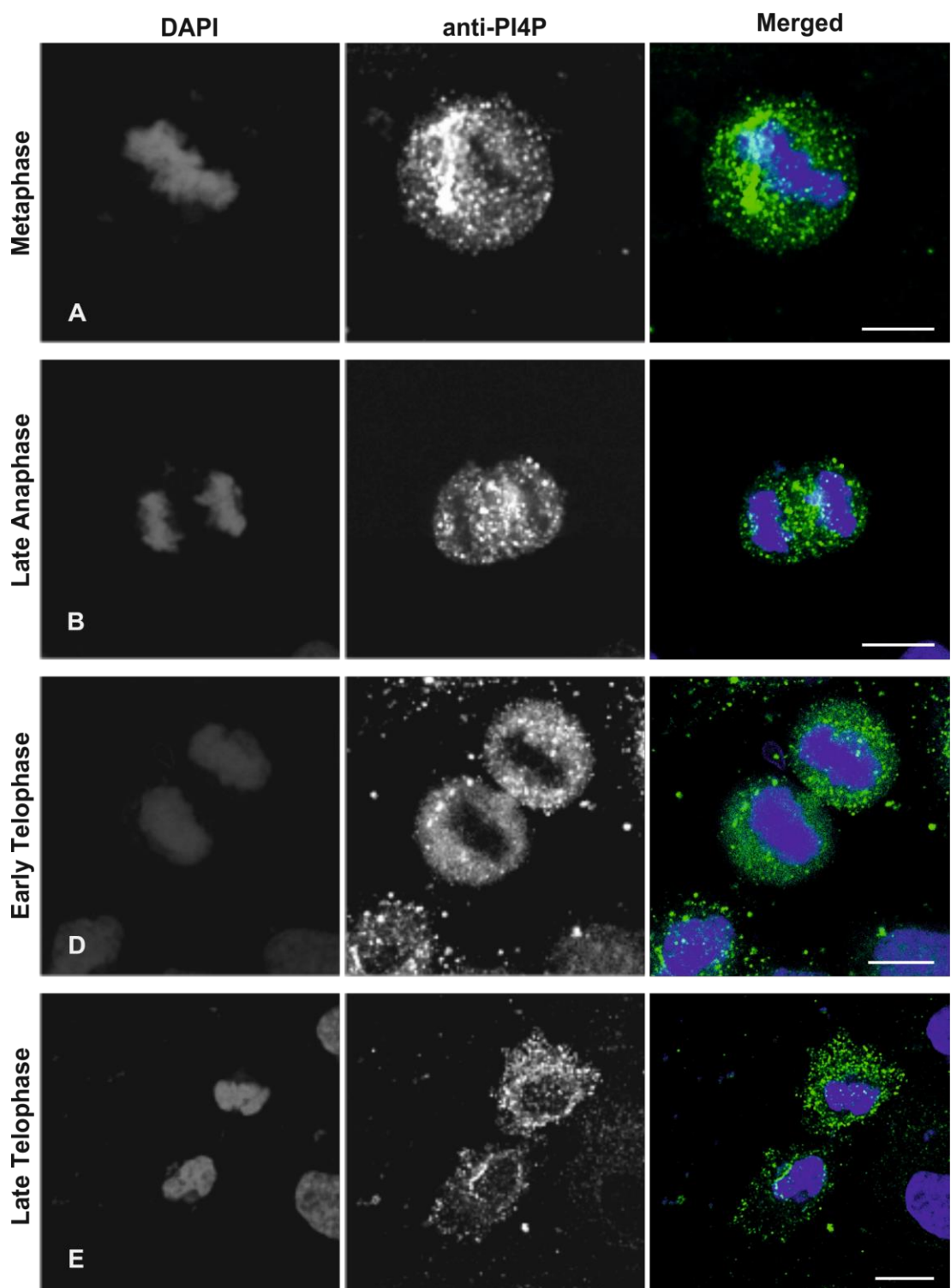


Figure 4.11. Distribution of endogenous PI4P during mitosis. HeLa cells stained for endogenous PI4P (green) and DAPI (blue) at defined stages of mitosis/cytokinesis as indicated on the left (A-D). Scale bars = 10 μ m.

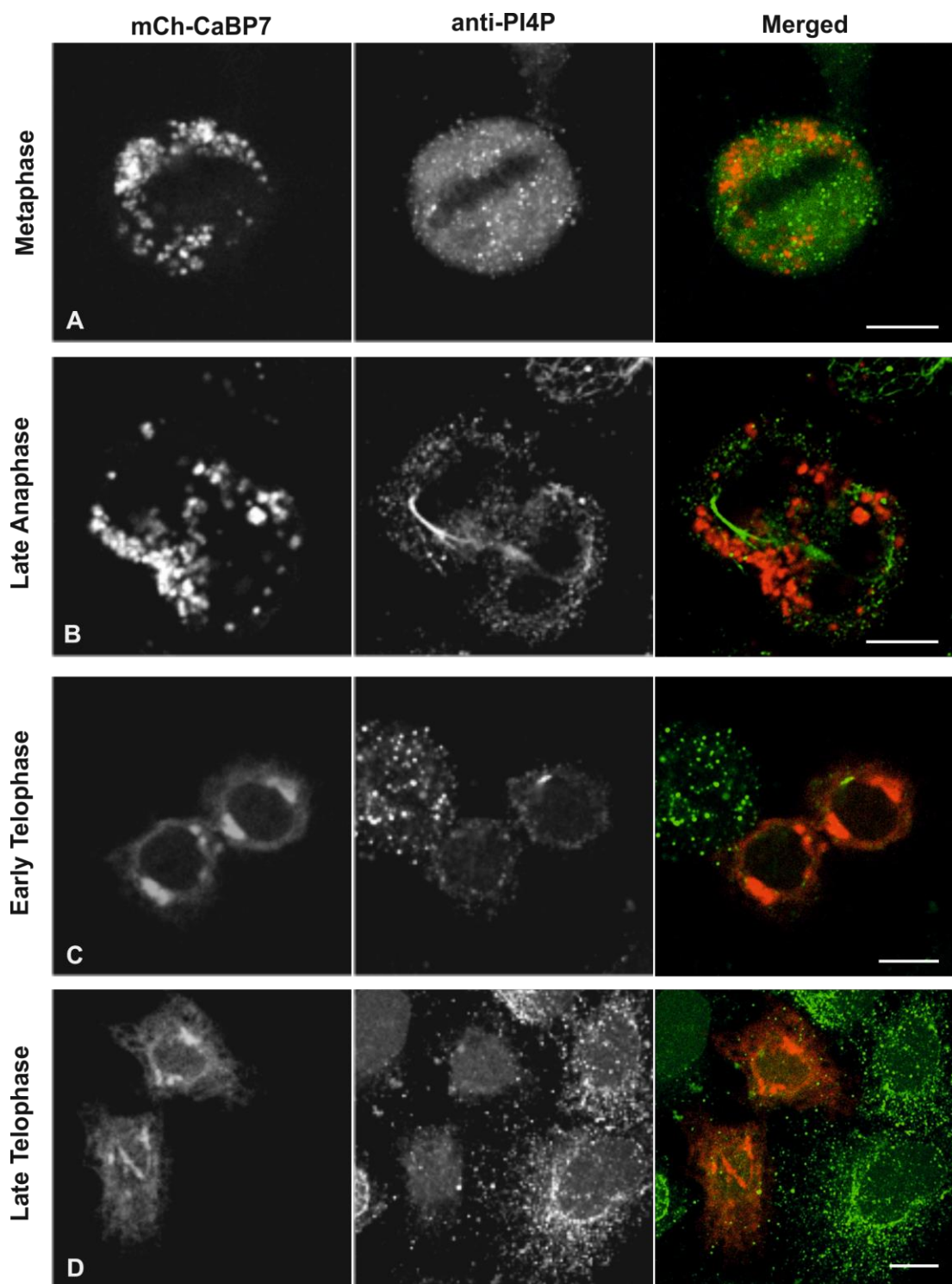


Figure 4.12. Distribution of endogenous PI4P during mitosis in cells overexpressing CaBP7. HeLa cells expressing CaBP7 (red) were stained for PI4P (green) at defined stages of mitosis/cytokinesis as indicated on the left (A-D). Scale bars = 10 μ m.

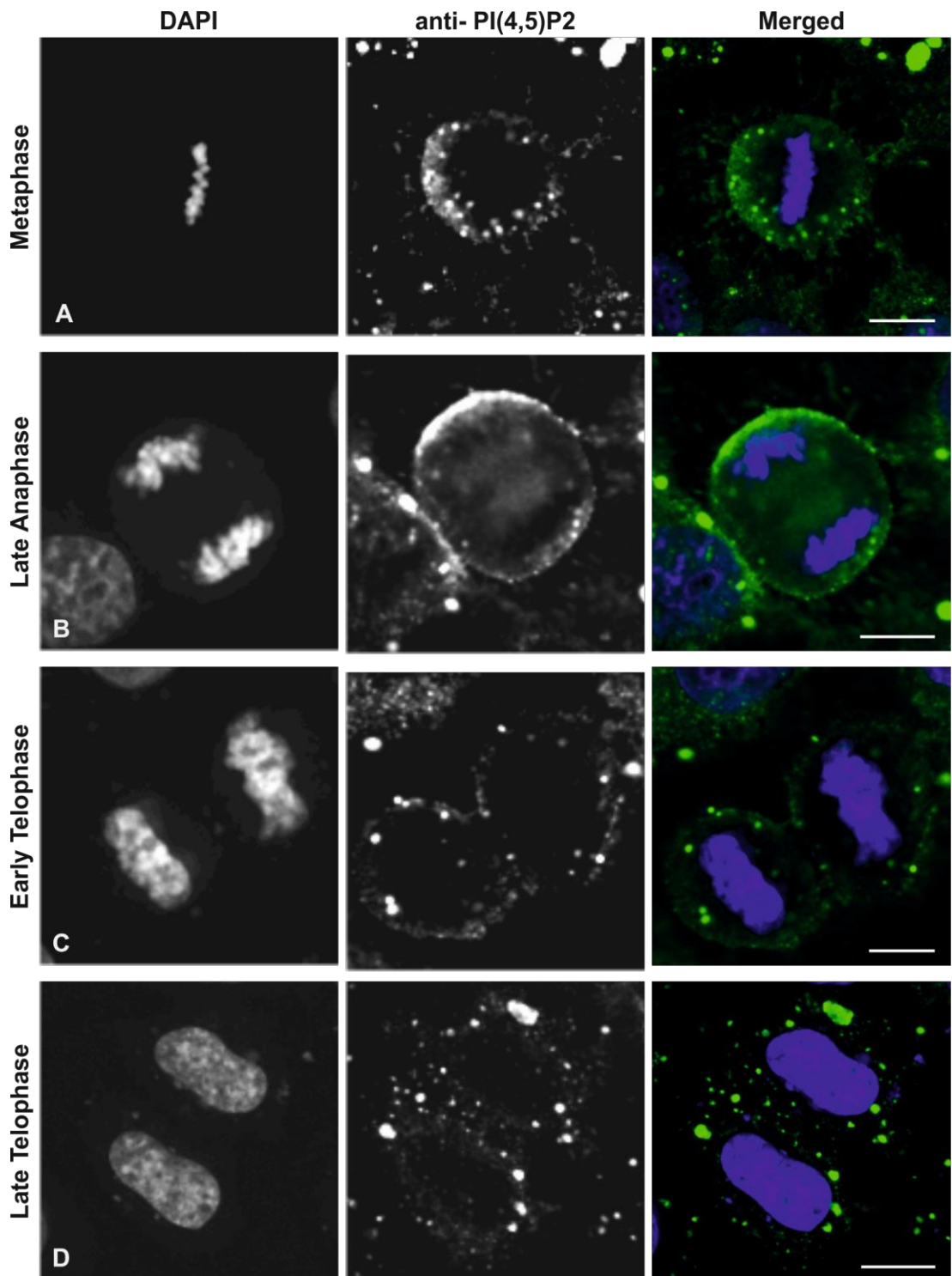


Figure 4.13. Distribution of endogenous PI (4,5) P₂ during mitosis. HeLa cells stained for anti-PI (4,5) P₂ (green) and nuclear stain DAPI (blue) at defined stages of mitosis/cytokinesis as indicated on the left (A-E). Scale bars = 10 μ m.

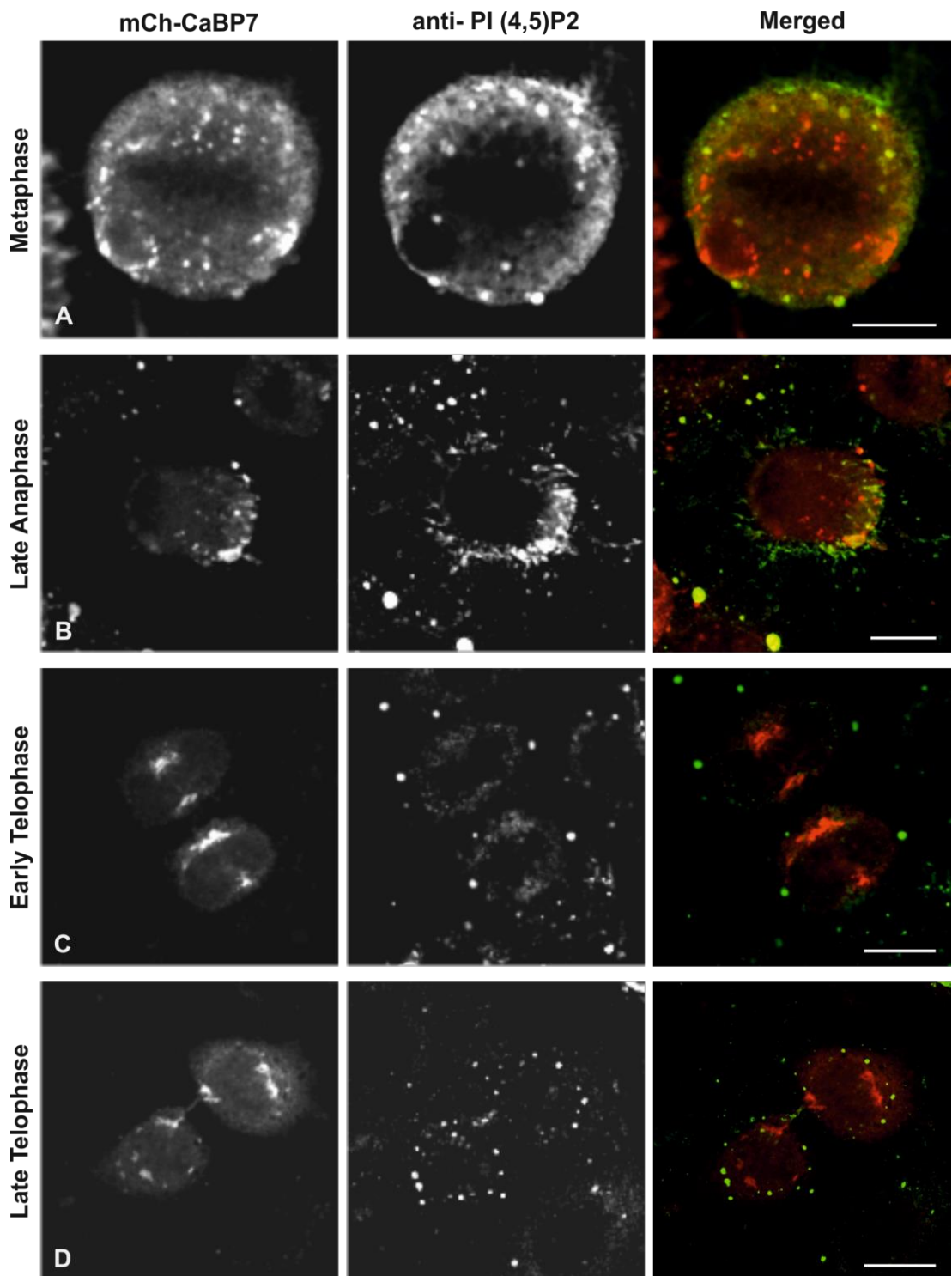


Figure 4.14. Distribution of endogenous PI (4,5) P₂ during mitosis in cells overexpressing CaBP7. HeLa cells expressing CaBP7 (red) were stained for anti-PI (4,5) P₂ (green) at defined stages of mitosis/cytokinesis as indicated on the left (A-D). Scale bars = 10 μ m.

4.2.7. Cell-cycle dependent redistribution of lysosomes is lost upon CaBP7 depletion

The data presented in this thesis have demonstrated that depletion of CaBP7 leads to cytokinesis failure and accumulation of binucleate cells possibly by loss of PI4KIII β inhibition. Furthermore, a novel redistribution and positioning of CaBP7, lysosomes and PI4KIII β during mitosis, which is particularly prominent at cytokinesis was observed. Lysosome clustering has previously been shown to be influenced by PI4KIII β activity (Chapter 3, figures 3.6-3.8) and importantly CaBP7 was able to specifically regulate lysosome distribution in these assays. Taken together these results suggest that CaBP7 could influence lysosome (and their resident PI4KIII β) trafficking during mammalian cell division. Therefore the critical question was whether CaBP7 depletion would impact on lysosome localisation during mitosis, in particular at telophase.

To address this, HeLa cells expressing the shRNAi plasmid targeting CaBP7 and control scrambled shRNAi were stained with tubulin and LAMP1 antibodies. Tubulin staining assisted in identifying cells that were at cytokinesis by the presence of the intercellular bridge. Indeed, CaBP7 down regulation appeared to impact on lysosome trafficking. Cells expressing shRNAi against CaBP7 displayed a loss of lysosome clustering to either side of the nuclei, whereas the control (scrambled shRNAi) cells still exhibited the previously characterised distribution of lysosomes during telophase (Figures 4.15. A and 4.5.). In order to quantify the loss of lysosome clustering on CaBP7 depletion, LAMP1 fluorescence intensity was quantified at the intercellular bridge regions between the control and CaBP7 knockdown

samples. A significant reduction of fluorescence intensity was seen in the CaBP7 knock down samples compared to controls (Figure 4.15. C), which mirrors the representative images (Figure 4.15. A). These results imply that depletion of CaBP7 and resulting failure of cytokinesis might be due to a lysosome trafficking defect.

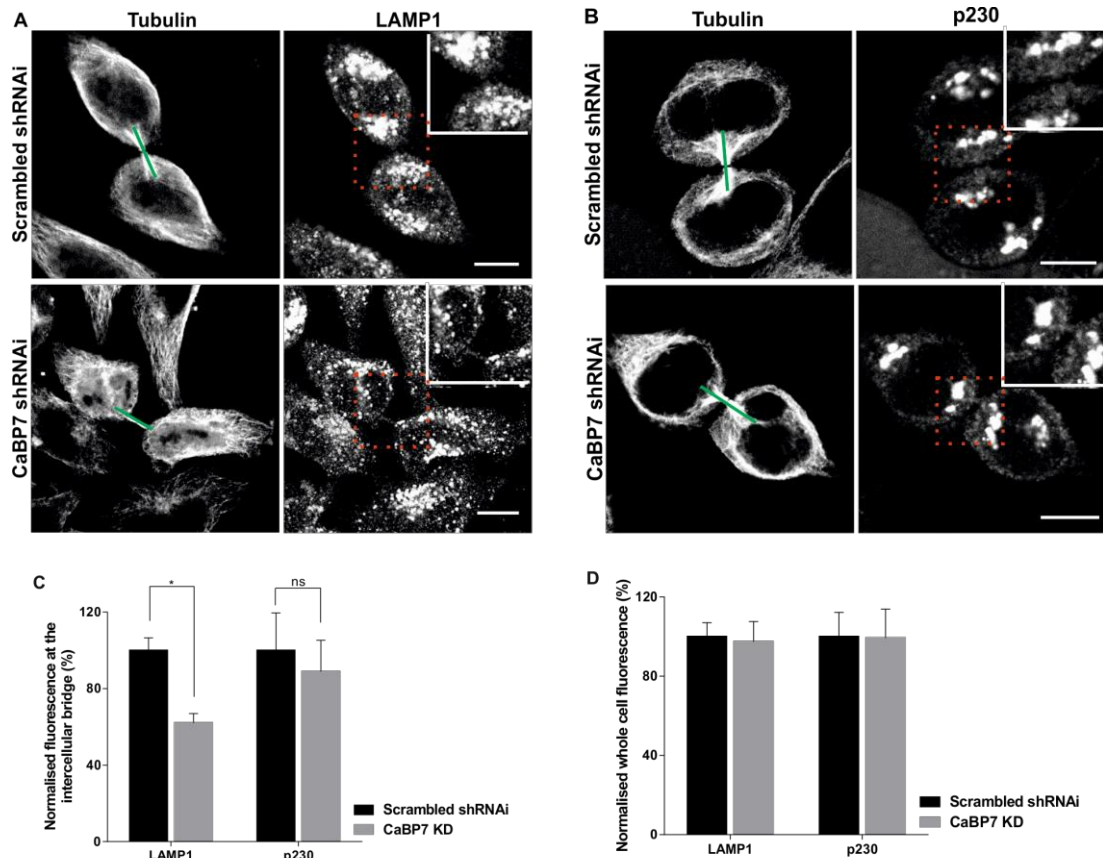


Figure 4.15. CaBP7 depletion and lysosome distribution during cytokinesis. A and B) Representative images of scrambled shRNAi and CaBP7 shRNAi transfected HeLa cells immunostained for endogenous LAMP1, p230 and tubulin. Cells at cytokinesis were imaged and LAMP1/p230 fluorescence intensity in the region spanning the intercellular bridge (green line in tubulin images) was quantified. Dashed red boxes highlight the zoomed regions displayed in the top right corner. Scale bars = 10 μ m. C) Normalised quantification data from A and B, D) Normalised quantification of total cell fluorescence. Unpaired t-test was performed (* $p < 0.05$), all data plotted \pm SEM.

In order to certify that the influence by CaBP7 depletion was specific to lysosome trafficking, positioning of the TGN was also assessed during cytokinesis. Cells expressing CaBP7 shRNAi and scrambled shRNAi were immunostained with antibody directed to the TGN specific marker p230. No difference in p230 staining at cytokinesis was detectable between scrambled shRNAi and CaBP7 shRNAi transfected cells (Representative images are shown in Figure 4.15. B). This observation was confirmed when data was quantified from a larger population of cells (Figure 4.15. C).

The observed loss of fluorescence intensity might be due to an overall reduction in lysosome numbers. Therefore it was imperative to analyse the total cellular LAMP1 fluorescence in all CaBP7 shRNAi transfected cells studied for the loss of mitotic lysosome redistribution. This quantification showed no difference in whole cell LAMP1 (or p230) fluorescence signal, indicating that CaBP7 does not influence total lysosome numbers (Figure 4.15. D).

Additionally, live- cell imaging of cells expressing the CaBP7 silencing construct and loaded with LysoTracker® were monitored through the cell cycle. CaBP7 depleted cells showed loss of lysosome redistribution and clustering during cytokinesis whereas in control (un-transfected) cells normal lysosome clustering was apparent (Figure 4.16.).

These investigations assisted in understanding the role of CaBP7 during cytokinesis and results obtained from this section provide new insights into the functional importance of CaBP7 during cell division.

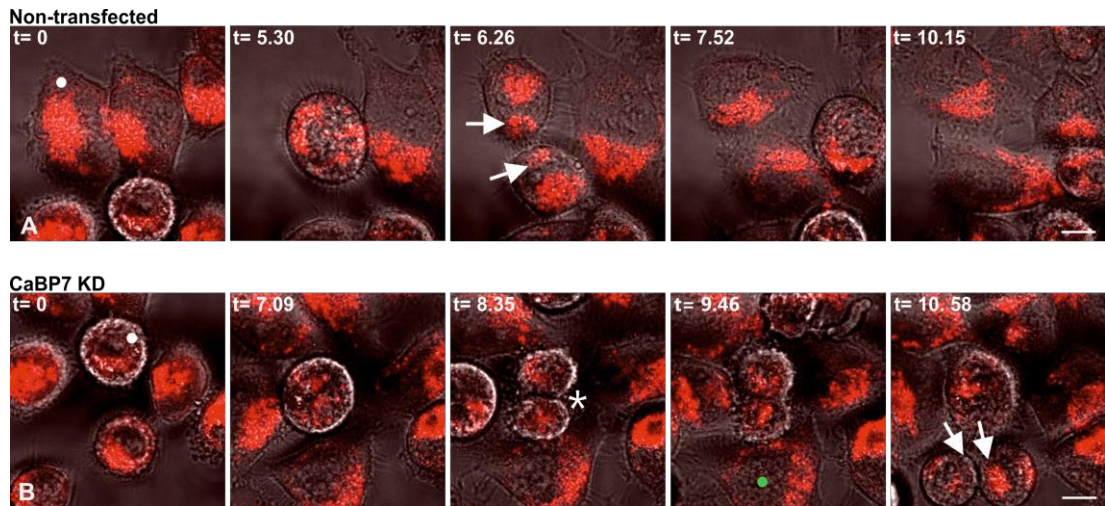


Figure 4.16. CaBP7 depletion and loss of lysosome clustering. HeLa cells expressing CaBP7 shRNAi were loaded with LysoTracker® and imaged overnight. Silencing constructs expressed GFP as a marker for transfection (not shown here). A) un-transfected mitotic cell; B) GFP positive cell expressing CaBP7 shRNAi at mitosis. White circle highlights cells that underwent mitosis, arrowheads depict lysosome clustering and asterisk marks loss of lysosome clustering. Green circle indicates another dividing control cell below the CaBP7 depleted cell. Imaging rate: 1 Frame/14.30 minutes, t= time in hours. Scale bars = 10 μ m.

4.2.8. CaBP7 depletion increases intercellular bridge lifetime at cytokinesis

Closer analysis of the live-cell videos showed that cells expressing the CaBP7 shRNAi construct exhibited a prolonged intracellular bridge lifetime (86.7 ± 9.0 minutes) compared to control (un-transfected cells), where the bridge lifetime was 25.2 ± 2.4 minutes (Figure 4.17.). These results suggest that CaBP7 depletion might increase intercellular bridge stability, which in turn might lead to cytokinesis failure and the formation of bi- and multi-nucleate cells.

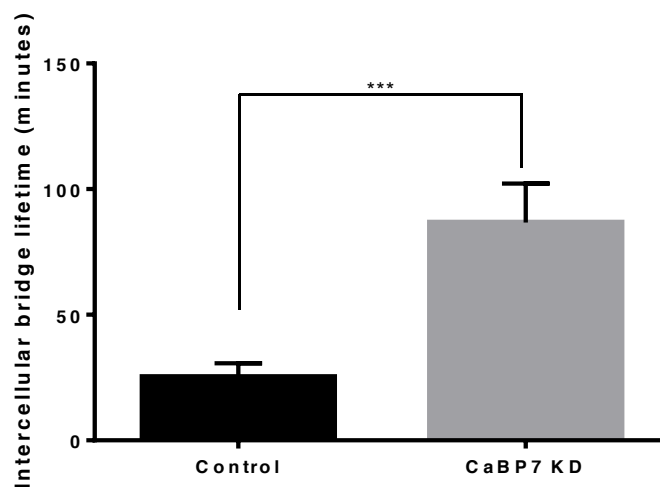


Figure 4.17. Intercellular bridge lifetime during cytokinesis. HeLa cells expressing scrambled shRNAi and CaBP7 specific shRNAi were analysed for the presence of the intercellular bridge during cytokinesis. Averaged data are plotted as a histogram \pm SEM. Unpaired t-test was performed (** $p < 0.001$).

4.3. Discussion

Mitosis is a fundamental process for complex multi-cellular organisms such as humans. It can be divided into various stages: Interphase, Prophase Prometaphase, Metaphase, Anaphase, Telophase and the final step Cytokinesis (Paweletz, 2001). The aim of successful mitosis is for a single mother cell to precisely copy its nucleus and the genetic content contained therein along with all its cellular contents so they can be divided into duplicate daughter cells. During cell division, three billion base pairs of DNA arranged over 23 pairs of chromosomes need to be copied without the introduction of potentially lethal mutations. To ensure error-free division numerous proteins are involved that check for correct completion of key

events and allow mitotic progression only if appropriate conditions have been satisfied. Even though mitosis has been studied in great detail, new studies constantly provide further understanding and insights into this highly organized process. In this thesis the primary focus is the very final stage of mitosis, cytokinesis, which culminates in the generation of new daughter cells. Neumann *et al.*, identified in their RNAi screen CaBP7 (amongst others) to play an essential role during cytokinesis (Neumann et al., 2010). CaBP7 deficient cells were competent to duplicate their nuclear content and successfully reached telophase ready to be physically split into two separate cells. Morphologically the two new daughter cells formed but remained connected by the intercellular bridge, which failed to undergo scission during cytokinesis. As a result of this, the daughter cells eventually coalesced back into a single entity that was now binucleate. If these binucleate cells underwent another round of cell division with cytokinesis failure, they became multinucleate. The presence of abnormal amounts of genetic material in a single cell (aneuploidy) is now an accepted driver of cellular transformation and tumorigenesis (Kops et al., 2005; Rajagopalan & Lengauer, 2004). After confirming the increased incidence of binucleate cells upon CaBP7 depletion (Figure 4.1.), studies were conducted to obtain further functional insights into how CaBP7 was operating during cytokinesis. As aforementioned, to date the only known interaction partner for CaBP7 is the lipid kinase PI4KIII β (Burgoyne & Haynes, 2010; Mikhaylova et al., 2009). CaBP7 is an inhibitor of PI4KIII β therefore depletion of CaBP7 protein will lead to loss of negative regulation of this kinase. To determine whether CaBP7 depletion-induced cytokinesis failure has a possible link to PI4KIII β regulation, this enzyme was

examined during mitosis. Indeed, enhanced PI4KIII β activity in cells provoked by PI4KIII β overexpression or overexpression of its activators, NCS-1 and Arf1, elicited an increased occurrence of binucleate cells (Figure 4.2.) mimicking results observed with CaBP7 depletion. This might suggest that unregulated activation of PI4KIII β is detrimental to cytokinesis completion. In agreement with this hypothesis the overexpression of a catalytically inactive form of PI4KIII β (PI4KIII β^{D656A}) as well as CaBP7 overexpression did not increase the number of binucleate cells above levels observed in control experiments (Figure 4.3.). Collectively these data imply that it is activation of PI4KIII β and not inhibition, which is detrimental to cytokinesis completion.

Consistent with the findings presented in this thesis, PI4KIII β has been implicated in previous studies as an important protein for successful cytokinesis in various model organisms (Polevoy et al., 2009; Rodgers et al., 2007), however it has not yet been studied in a mammalian system. In *Drosophila* the PI4KIII β homologue Fwd was identified as a key regulator of cytokinesis in spermatocytes but not somatic cells (Polevoy et al., 2009). In this study the importance of the synthesis of PI4P by PI4KIII β during cytokinesis was described. The catalytically inactive version, PI4KIII β^{D656A} , partially rescued cytokinesis failure in *fwd* knockout flies, suggesting a non-catalytic function of PI4KIII β during cytokinesis. It was further demonstrated that the small GTPase Rab11 is recruited to the midzone by PI4KIII β during cytokinesis (Polevoy et al., 2009), and that there was an aberrant localisation of Rab11 in *fwd* flies. PI4KIII β function has also been studied in the trypanosome parasite *T. brucei* (*Tb*). *Tb* PI4KIII β has many vital functions

including Golgi structure maintenance, protein trafficking, regulation of cellular shape and cytokinesis (Rodgers et al., 2007). Depletion of *Tb*-PI4KIII β led to cell growth inhibition and a defect in cell cycle progression (Rodgers et al., 2007). These data underline the importance of PI4KIII β for successful mitosis completion.

The results presented here on PI4KIII β contradict the published data from model organisms. In this thesis the over activation of PI4KIII β rather than the loss of activity caused a cytokinesis defect. Yet the published studies showed non-catalytic in addition to catalytic functions of PI4KIII β , hence, even though PI4KIII β is required, its over activation can be similarly detrimental. The requirement for PI4KIII β activity during mammalian cell mitosis has yet to be studied and data presented here provides initial evidence that it does exert an important function.

To further understand CaBP7's role in mitosis, its distribution during cell division was examined. Strikingly CaBP7 displays a distinctive localisation during cell cycle stages especially at telophase where it clusters to either side of the nuclei (Figure 4.5. E, F). To verify whether this clustering occurs along with lysosomes or the TGN (the two major sites of CaBP7 localisation), cells were stained with lysosome markers (LAMP1 and CD63, Figure 4.6. 4.7. 4.8.) and a TGN marker (p230, Figure 4.9.) along with CaBP7. Detailed analysis of the co-localisation of these markers with CaBP7 revealed that CaBP7 switches from a predominantly TGN association at interphase to the lysosomes during cell cycle progression with the greatest lysosomal co-localisation coinciding with telophase (Table 4.2.). These results propose not

only CaBP7 to have essential roles during mitosis but that lysosomes may also exert unique functions during cytokinesis.

Little attention has been given to lysosomes during cell division. Bergeland *et al.*, examined the fate of endosomes and lysosomes during mitosis and their results revealed that both organelles cluster to the microtubule organization centre during cytokinesis (Bergeland, Widerberg, Bakke, & Nordeng, 2001). However localisation analysis of lysosomes and CaBP7 performed in this thesis show lysosomes to localise and cluster near the intercellular bridge at cytokinesis, opposite to the site of the cellular poles. A similar cell-cycle dependent redistribution of lysosomes has been previously observed but not elaborated upon (MATTEONI & KREIS, 1987). In contrast, the contribution of endosomes to mitosis has been studied in greater depth. Endosomes have been proposed to serve as membrane reservoirs during the onset of anaphase, where the cleavage furrow requires additional membrane to form the cytokinetic bridge between daughter cells (Boucrot & Kirchhausen, 2007; Goss & Toomre, 2008). The delivery of extra membrane to permit cytokinesis completion could also be facilitated by the exocytosis of lysosomes. Lysosomes have been established to act as membrane donors in membrane repair mechanisms (Idone et al., 2008; McNeil, 2002; A. Reddy et al., 2001), therefore it is possible that lysosomes might also fuse with the plasma membrane during the final stages of cytokinesis.

To investigate further functional links between CaBP7, PI4KIII β and lysosomes in cytokinesis, the positioning of PI4KIII β during cell cycle stages was also studied. PI4KIII β displayed a similar redistribution to CaBP7 and showed the same increased co-localisation with LAMP1 (lysosomes) at

telophase (Figure 4.10. C, D). This observation is consistent with a role for PI4KIII β during cytokinesis and hints that the lysosomal pool of PI4KIII β is positioned to play a role during cytokinesis. Work from the previous chapter of this thesis revealed insights into CaBP7 function on lysosomes. The results showed that CaBP7 likely regulates the lysosome resident PI4KIII β pool. Taken in their entirety, these results are consistent with a CaBP7 function on lysosomes, which could be to regulate PI4KIII β activity during mitosis. CaBP7 overexpression inhibited PI4KIII β and reduced PI4P levels as shown in Chapter 3. Here these studies have been extended to assess whether this can be seen throughout cell division or whether it is limited to interphase. Conceptually, this is an important issue since the working hypothesis is that CaBP7 functions during cytokinesis by regulating lysosomal PI4KIII β to modulate local levels of PI4P.

PI4P levels were observed throughout the cell cycle in a background of CaBP7 overexpression. Results revealed that CaBP7 overexpression caused a visible reduction of PI4P level compared to control cells at all stages of mitosis and cytokinesis (Figures 4.11. and 4.12.). PI4P is the main precursor of the major phosphoinositide signalling species PI (4,5) P₂, and perturbation in PI4P levels could conceivably impact on PI (4,5) P₂ synthesis. No obvious difference in PI (4,5) P₂ levels could be detected however between control and CaBP7 overexpressing cells at any cell cycle stage (Figure 4.13. and 4.14.). It should be noted that overexpression of CaBP7 and PI4P depletion do not induce cytokinesis failure but these results do show that CaBP7 is functionally capable of modulating PI4P production during cell division.

Under normal cellular conditions with endogenous levels of CaBP7 it is entirely possible that regulation of PI4P levels during cell division is intimately linked to PI (4,5) P₂ signalling and there have been a number of studies documenting a role for PI (4,5) P₂ during cytokinesis and abscission (severance of the intercellular bridge to generate two independent daughter cells). It has been demonstrated that the dynamic production and hydrolysis of PI (4,5) P₂ is imperative for successful cytokinesis. Local synthesis of PI (4,5) P₂ is of functional importance during intercellular bridge assembly and in determining subsequent bridge stability (Chesneau et al., 2012; Kouranti, Sachse, Arouche, Goud, & Echard, 2006). Conversely, depletion of PI (4,5) P₂ is required for successful abscission. These phenomena were studied by Dambournet *et al.*, through depletion of the PI (4,5) P₂ 5- phosphatase OCRL (oculocerebrorenal syndrome of Lowe) which led to the formation of very stable intercellular bridges with major delays in, or complete inhibition of, abscission (Dambournet et al., 2011). Quantification of PI (4,5) P₂ levels in the stable bridges of OCRL depleted cells highlighted an increased amount of PI (4,5) P₂ compared to controls. Additionally, F-actin accumulation was also observed in these bridges, consistent with the fact that PI (4,5) P₂ controls F-actin polymerization. These analyses demonstrated the importance of tightly regulated, dynamic, lipid synthesis and hydrolysis throughout mitosis to permit normal progression and successful completion of cell division (Echard, 2012; R. Wong et al., 2005). The cytokinesis defect documented in this thesis chapter, could potentially be due to the accumulation of PI (4,5) P₂ throughout the final stages of mitosis, however the exact explanation of why excessive PI (4,5) P₂ or F-actin inhibit

abscission has not yet been determined. One possibility is that the excessive accumulation of these molecules might prevent proper assembly, localisation and constriction of the ESCRT III machinery which is established to be critical for abscission (Elia et al., 2011; Lafaurie-Janvore et al., 2013). Moreover, an abnormal lipid composition in cellular membranes important during cytokinesis could lead to other complications. For example, excess amounts of PI4P or PI (4,5) P₂ could lead to sequestration of key proteins which are perhaps required elsewhere. A second possibility is that accumulation of these PI4P and PI (4,5) P₂ might result in excessive positive membrane curvature which could prevent successful cytokinesis (Dambournet et al., 2011).

The data presented in this thesis chapter indicate that regulation of PI4P levels by CaBP7 and PI4KIII β are important during mammalian cell cytokinesis however a precise molecular understanding of how PI4P influences this process is an outstanding question. Further experiments examining changes of PI4P and PI (4,5) P₂ levels need to be undertaken to understand the specific defect. To address this issue, the key experiment will be to monitor PI4P and PI (4,5) P₂ levels upon CaBP7 depletion during cell division. The expectation would be that levels of PI4P and perhaps PI (4,5) P₂ would be increased under such conditions which would be consistent with the reported influence of PI (4,5) P₂ on cell division as discussed above. Interestingly, intercellular bridge stability is increased in CaBP7 depleted cells as described in this thesis chapter which is the same phenotype observed when hydrolysis of PI (4,5) P₂ is prevented.

It would also be interesting to examine whether there are PI4KIII β independent functions of CaBP7 during mitosis. CaBP7 regulates the kinase independently of Ca²⁺ and the possibility exists that it has additional Ca²⁺ dependent roles to play. In order to examine this PI4KIII β could be depleted from cells or newly documented PI4KIII β specific inhibitors could be employed (Mejdrová et al., 2015).

Further insights into how CaBP7 depletion influences cytokinesis were gained with the observation that normal lysosome clustering at cytokinesis was lost upon CaBP7 down regulation (Figure 4.15.). In CaBP7 depleted cells at cytokinesis lysosomes appeared disperse throughout the cell and did not localise tightly in clusters to the intracellular bridge region and poles, compared with control cells (Figure 4.15.). These findings clearly show that CaBP7 knockdown influences lysosome trafficking during cytokinesis, which in turn might explain the cytokinesis failure. CaBP7 influence on PI4P levels and subsequent lysosome trafficking alteration during cytokinesis is summarized in Diagram 4.2. as an overview functional model for the collective data presented in this chapter.

Ultimately the question arises: What is the role of lysosomes during cell division? To date the potential role of lysosomes during mitosis has been overlooked in favour of studies focusing on the roles of early- and recycling-endosomes, as briefly described above. Recycling endosomes have been implicated in various studies as key players in abscission (Ai & Skop, 2014; Montagnac & Chavrier, 2008; Schiel & Prekeris, 2010).

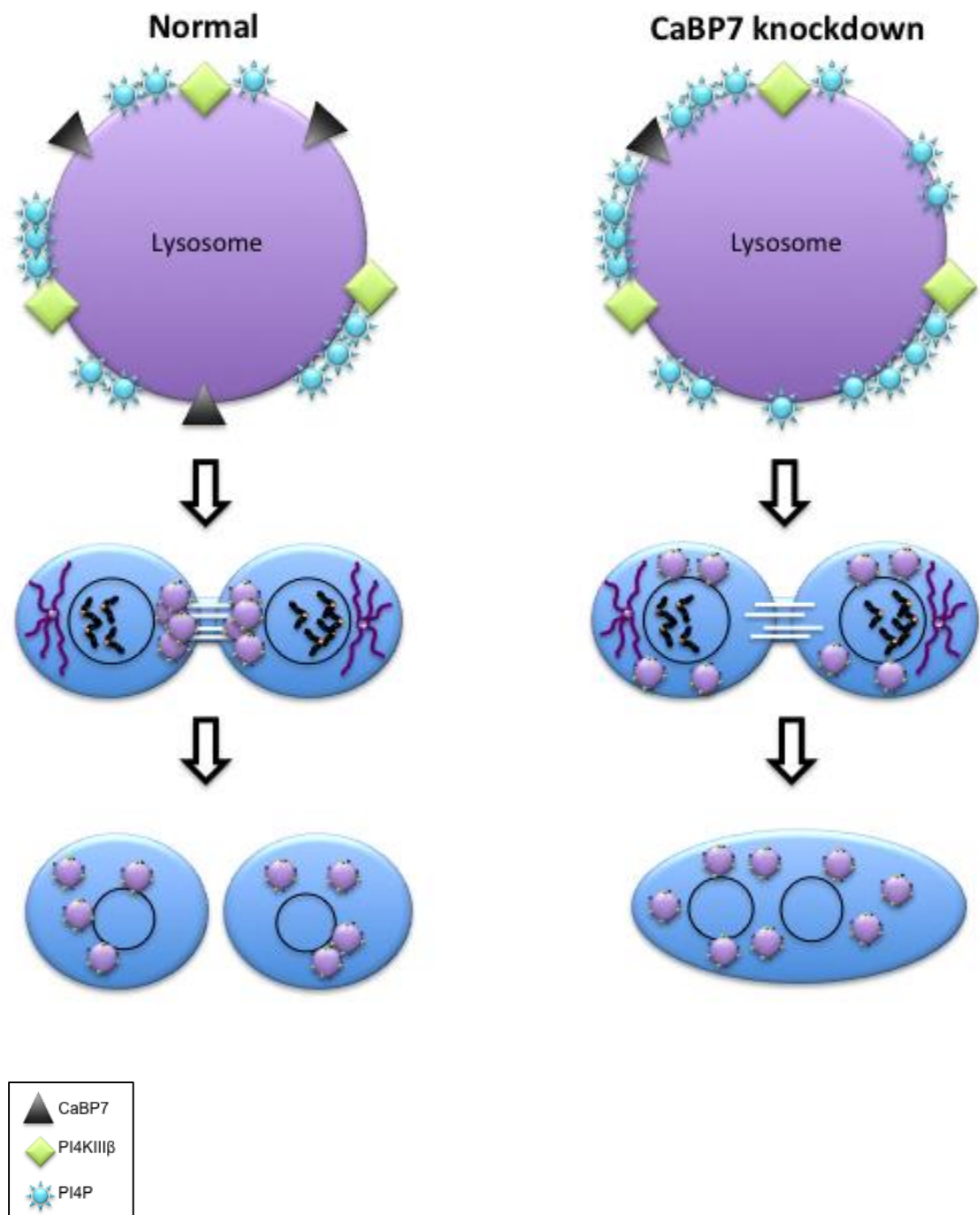


Diagram 4.2. Functional model of the effect of CaBP7 on cytokinesis. PI4KIIIβ on the lysosomes will be basally active upon CaBP7 knockdown, producing increased levels of PI4P. This might prevent lysosome clustering at the sides of the intercellular bridge and ultimately causes cytokinesis failure.

Rab11, a small GTPase trafficking recycling endosomes during interphase, has been shown to be targeted to the cleavage furrow during cytokinesis (G. M. Wilson et al., 2005).

Normal Rab11 function is essential for cytokinesis completion in *C. elegans* (Skop, Bergmann, Mohler, & White, 2001), *Drosophila* (Hickson et al., 2003; Riggs et al., 2003) and also in mammalian cells (Takahashi et al., 2011; G. M. Wilson et al., 2005; G. Wilson et al., 2004). Rab11 specifically recruits FIP3 (Family of interaction protein- 3) to the cleavage furrow and this Rab11-FIP3 complex is essential for abscission, proving the importance of recycling endosomes in this final step of cell division (Takahashi et al., 2011; G. M. Wilson et al., 2005). Additionally Rab35, another small GTPase, has also been identified as a key player for cytokinesis by localising specific Septins to the intercellular bridge in mammalian cells (Chesneau et al., 2012; Kouranti et al., 2006).

In light of recent studies expanding on the known functions of lysosomes and data presented in this thesis documenting a connection between lysosome trafficking, CaBP7 and PI4KIII β during mammalian cell cytokinesis a new examination of lysosome function during mitosis is timely. Lysosomes might have degradative functions during cytokinesis. Cytokinesis is a complex process, which can be further divided into stages, including the assembly of the cytokinesis apparatus, furrow ingression and abscission (Barr & Gruneberg, 2007). During these steps there is a requirement for the intercellular bridge to be removed or degraded followed by extensive remodelling of the plasma membrane (Barr & Gruneberg, 2007; Fededa & Gerlich, 2012; Schiel & Prekeris, 2010). Since this study and that of Metteoni

and Kreis and have shown that lysosomes localise closely to the edges of the intercellular bridge it could be that the degradation of this cytokinesis specific structure is performed by lysosomes (MATTEONI & KREIS, 1987). One possibility is that the correct localisation of lysosomes perturbed upon CaBP7 knockdown, prevents bridge removal and consequently cells remain connected and eventually merge back into a single cell. This would be consistent with the results of Neumann *et al.* where time resolved microscopy clearly showed CaBP7 depleted cells reaching cytokinesis but being unable to successfully separate. These cells eventually coalesced back into a single cell containing two nuclei (Neumann *et al.*, 2010). This hypothesis of lysosome activity is similarly consistent with the observation from this thesis of the prolonged intercellular bridge lifetime in CaBP7 depleted cells (Figure 4.17.). The analysed data showed cells failing mitosis as a consequence of a cytokinesis defect where the nascent daughter cells failed to separate (extended bridge lifetime) and ultimately became a single binucleate cell.

Another possible requirement of lysosomes during cytokinesis might be as membrane donors similar to the published function of endosomes. Lysosomes have been studied extensively for their function in plasma membrane repair (Idone *et al.*, 2008; McNeil, 2002; A. Reddy *et al.*, 2001). In those studies, scratch assays were employed to wound cells and evaluate the appearance of the luminal domains of lysosome resident proteins at the cell surface which would indicate fusion of these organelles with the PM. Results revealed a Ca^{2+} dependent lysosome exocytosis machinery that mediated fusion with the PM in order to effect a repair of the membrane damage. These data coupled to observations of lysosome clustering during

cytokinesis and a known requirement for membrane donation during cytokinesis would certainly make lysosome fusion a plausible potential function.

Lysosomes are now recognised as important Ca^{2+} storage organelles (A. J. Morgan, Platt, Lloyd-Evans, & Galione, 2011). The extraordinary localisation of lysosomes along with CaBP7 during cytokinesis might also reflect a requirement for localised Ca^{2+} signalling. Several Ca^{2+} channels are present on the lysosomal membrane including the TRP-Mucolipin subfamily (TRPML) and the Two- pore channels (TPCs). TRPML comprises three members, TRPML1, TRPML2 and TRPML3 that are non-selective Ca^{2+} channels which are all found on the lysosomes, however TRPML2 and TRPML3 are also present on recycling and early endosomes (X. Cheng, Shen, Samie, & Xu, 2010; Karacsonyi, Miguel, & Puertollano, 2007; H. J. Kim, Soyombo, Tjon-Kon-Sang, So, & Muallem, 2009). TPCs are a novel family of intracellular Ca^{2+} release channels exclusively localised to the endo-lysosomal system (Galione et al., 2010) and thought to be activated by the potent second messenger nicotinic acid- adenine dinucleotide phosphate (NAADP). The importance of lysosomal Ca^{2+} signalling in numerous cellular processes is now established (A. J. Morgan et al., 2011). Explicit functions for Ca^{2+} transients originating from lysosomes have been demonstrated during autophagy for instance. Autophagy allows the degradation and recycling of cell components ensuring cell homeostasis. Ca^{2+} signalling initiating from lysosomal TRPML1 controls autophagy by regulating calcineurin- mediated stimulation of transcription factor EB (TFEB), a master transcriptional regulator of autophagy. Activated calcineurin dephosphorylates TFEB

allowing its nuclear translocation (Medina et al., 2015). Another example of lysosomal Ca^{2+} function was established in immune cell physiology. Cytotoxic T-lymphocytes (CTLs) destroy infected and tumorigenic cells by the release of cytolytic proteins (granzymes and perforin). The mobilisation of acidic (lysosomal) Ca^{2+} stores by NAADP contributed to T-cell receptor stimulation and drives exocytosis of cytolytic factors from CTLs (Davis et al., 2012). Ca^{2+} transients have been suggested as potentially important during mammalian cell mitosis, although the exact nature of such Ca^{2+} signals and their mechanism of action remains to be determined (Whitaker, 1997; 2006). One possibility consistent with the data presented in this thesis is that localised Ca^{2+} signalling via lysosome specific micro domains could be necessary to allow downstream signalling pathways and progression through the cell cycle. CaBP7 as a Ca^{2+} sensor protein, localised to the lysosomes, might conceivably play a role in trafficking lysosomes correctly during cytokinesis to allow essential Ca^{2+} release from lysosomes in a spatio-temporally regulated manner. This would be consistent with findings that depletion of TPC1 and TPC2 (discussed above) also caused cytokinesis defects in the same study that uncovered a role for CaBP7 during cytokinesis (Neumann et al., 2010).

At this stage it is not possible to explain the exact mechanism of the cytokinesis defect elicited by CaBP7 loss of function although control of phosphoinositide metabolism via PI4KIII β is certainly part of the story. Lysosomes have been clearly neglected in studies of cell division and further work will be needed to examine CaBP7 and lysosome functions in greater detail during this process.

4.4. Summary

This chapter investigated the function of CaBP7 during mitosis, in particular at cytokinesis. The depletion of CaBP7 as well as the activation of PI4KIII β led to a significant increase in the occurrence of binucleate cells. Examining the distribution of CaBP7 during cell cycle progression revealed a distinctive clustering phenotype along with lysosomes during the later stages of mitosis. A similar redistribution was observed for PI4KIII β , inferring CaBP7, lysosomes and PI4KIII β to have a linked function in cytokinesis. CaBP7 was demonstrated to be capable of regulating PI4KIII β throughout the process of cell division. Further analysis on CaBP7 depletion during cytokinesis illustrated a loss of normal lysosome clustering in addition to increased intercellular bridge stability/ lifetime at cytokinesis, which could contribute to the observed failure of cytokinesis.

Chapter 5

Investigating lysosome function during mitosis

5.1. Introduction

Mitosis encompasses a series of intricate molecular events leading to cellular duplication through division of a progenitor mother cell into two identical daughter cells. The molecular mechanisms and processes that ensure high fidelity during DNA replication and subsequent segregation of chromosomes between the daughter cells have been researched extensively (Malumbres & Barbacid, 2009). Mitosis can be divided into four major phases: prophase, metaphase, anaphase and telophase followed by cytokinesis. In prophase the nuclear envelope breaks down and the replicated chromatin is loosely condensed into chromosomes. During metaphase chromosomes align along the mitotic plate and can attach to the microtubules and the centrosomes. Centrosomes relocate to the opposite spindle poles, and chromatids travel along the microtubules to each pole at anaphase. At telophase the nuclear envelope reforms around the new DNA pools and eventually at cytokinesis cells are physically separated into two entities (Leman & Noguchi, 2014). Detailed analysis of this process has revealed the requirement of various core proteins including the cyclins and cyclin-dependent kinases (Cdks) to ensure precise duplication and segregation of genomic DNA during mitosis (Hartwell & Weinert, 1989; Kastan & Bartek, 2004; Vermeulen et al., 2003). However equally important is the segregation of intracellular organelles. For the survival of the daughter cells it is imperative they inherit cellular organelles to process the information encoded by the genome (Y. Imoto, Yoshida, Yagisawa, Kuroiwa, & Kuroiwa, 2011; Jongsma, Berlin, & Neefjes, 2015). Single- and double- membrane bounded organelles have to be accurately segregated into the new cells, since these cannot be synthesised

de novo (Y. Imoto et al., 2011). The division of organelles occurs in a sequence of events subject to exact spatio-temporal control. In addition to a requirement for sub-cellular organelles to be partitioned into the new daughter cells, some also play an active role during mitosis to provide specific essential functions required for successful completion of cell division. Nuclear membrane break down and reformation are two essential signals for the progression of mitosis. At prometaphase the disassembly of the nuclear membrane permits access of the developing spindle to the chromosomes. The other crucial role is to protect and keep the condensed DNA separate from the cytosol as in interphase and therefore the nuclear envelope rebuilds at late telophase when chromosomes begin to compress.

Mitochondria are the main powerhouse of the cell and this energy yielding functionality is key during the dynamic process of mitosis. New daughter cells also have an absolute requirement for inherited mitochondria to fuel on going metabolic processes following cell division. The activity of mitochondria in cell division is quite complex as these organelles contain their own genomic DNA (mtDNA), which encodes crucial bioenergetics information. Therefore daughter cells must obtain correctly segregated mitochondria as well as the mtDNA. There is a highly complex molecular mechanism in place to ensure proper partitioning of mitochondria during cell division. Mitochondria undergo a fusion- fission cycle and interact with the cytoskeleton (through molecular motor proteins) and ER elements to ensure equal segregation into daughter cells (Mishra & Chan, 2014).

The Golgi apparatus displays a very unique and exceptional role during mitosis. The Golgi complex is structured as a continuous system of

membranous stacks or cisternae, which are connected by tubular bridges (Rambourg, Clermont, Hermo, & Segretain, 1987). This organelle is localised to the perinuclear region and has critical biosynthetic functions including: Protein/lipid trafficking, lipid biosynthesis, protein modification and the transport and sorting of proteins to particular locations along the secretory pathway (Marsh & Howell, 2002). During cell division the Golgi fragments in a multistage process that ensures correct inheritance to the daughter cells (Colanzi, Sutterlin, & Malhotra, 2003a; Shorter & Warren, 2002). However not only is the extensive fragmentation essential for partitioning into new cells, but also for allowing mitotic entry and therefore cell cycle progression (Sutterlin, Hsu, Mallabiabarrena, & Malhotra, 2002). The progressive disassembly of the Golgi serves as a Golgi mitotic checkpoint, signalling the cell to progress to the next stage. By inhibiting proteins involved in Golgi break down such as GRASP-65 and CtBP1-S/BARS, cell cycle was arrested at G2 phase and cells failed to enter mitosis (Colanzi et al., 2007; Hidalgo Carcedo et al., 2004; Preisinger et al., 2005; Yoshimura et al., 2005). Furthermore depleting mitogen activated protein kinases (MAP kinases) such as RAF1, MEK1, ERK1 (components regulating Golgi disassembly) also led to a delay in the G2/M transition (U. Acharya, Mallabiabarrena, Acharya, & Malhotra, 1998; Colanzi, Sutterlin, & Malhotra, 2003b; Shaul & Seger, 2006). These studies clearly demonstrate that mitotic fragmentation of the Golgi apparatus is required for daughter cell inheritance and that this event regulates the cell cycle such that any blockade or failure in fragmentation inhibits mitotic progression.

The endoplasmic reticulum (ER) has also been investigated during mitosis. Confocal and electron microscopy (EM) studies revealed that the ER, as a dynamic organelle, displays visible morphological changes through cell division. The ER can be subdivided into three domains: 1) The nuclear envelope; 2) The rough ER (RER) that is decorated with bound ribosomes translating secretory pathway proteins and 3) The smooth ER (SER) which harbours specialised sites of transport vesicle biogenesis for the trafficking of protein and lipid cargo out of the ER. The RER and SER are spread throughout the cytoplasm and make extensive contacts with other organelles (Voeltz, Rolls, & Rapoport, 2002). As described earlier, the nuclear envelope plays an indispensable role during mitosis, therefore the ER is directly involved in mitotic progression (Ellenberg et al., 1997). Furthermore the ER in flies has been shown to be the source of localised calcium (Ca^{2+}) signalling activity during mitosis (Parry, McDougall, & Whitaker, 2005). The implication of ER Ca^{2+} signalling in cytokinesis has further been shown by studying IP_3R and IP_3 in the fish *Danio rerio* (K. W. Lee et al., 2003; W. M. Li et al., 2008). The ER also functions in the biosynthesis and transport of phospholipids (Fagone & Jackowski, 2009). A role for the ER as provider of phospholipids during the extensive membrane restructuring that occurs during mitosis is a theoretical possibility, which has yet to be evaluated experimentally.

Endosome segregation and partitioning during mitosis remains unclear although some studies have observed clustering of these organelles around centrosomes which could be of physiological relevance (Takatsu et al., 2013). Functionally, during mitosis, endosomes have been shown to provide additional membrane material at anaphase to establish the cleavage furrow

which in turn permits formation of the cytokinetic bridge (Arden, Puri, Au, Kendrick-Jones, & Buss, 2007; Boucrot & Kirchhausen, 2007; Goss & Toomre, 2008; G. M. Wilson et al., 2005). Endosomes have additionally been implicated in regulating the assembly and delivery of abscission machinery (Schiel et al., 2012).

As discussed above, a functional requirement for many of the different organelles present in mammalian cells has been established during mitosis with the exception of lysosomes. Bergeland *et al.*, in an effort to study endosomes and lysosomes during mitosis demonstrated that lysosomes remain intact and segregate into daughter cells by a coordinated movement during cytokinesis, although partitioning was not completely accurate (Bergeland et al., 2001). However, in this study, lysosomes were shown to accumulate in a perinuclear region and not at the intercellular bridge area as reproducibly observed in the previous results chapter of this thesis and in the work of Matteoni and Kreis (MATTEONI & KREIS, 1987). To date, there is no published data examining the potential functional importance of lysosomes during mitosis in mammalian cells or other model organisms. Although catabolism of cellular debris is a key activity of lysosomes, a view of these unique organelles simply as waste disposal sites of the cell has now been revised. Various studies have revealed multiple non-degradative lysosome functions involved in many normal cellular activities. In light of these findings lysosomes are now recognised as essential regulators of cellular homeostasis. As aforementioned, the most well-known and well-studied function of lysosomes is in the degradation of macromolecules of intra- or extra-cellular origin (Appelqvist et al., 2013). During mitosis certain dynamic

and temporary cellular structures are created that require removal for the effective completion of cell division. Therefore degradative properties of lysosomes might be required, for instance, during late telophase in the process of abscission machinery break down and removal. Additionally, lysosomes could play a role as membrane donors during mitosis in a manner analogous to that reported for endosomes (Boucrot & Kirchhausen, 2007). Lysosomal exocytosis with the plasma membrane (PM) has been studied in models of PM wound repair and similar events might occur during the complex membrane restructuring processes required during mitosis and especially at later stages, where additional membrane has to be incorporated into the PM to permit daughter cell separation (McNeil, 2002; A. Reddy et al., 2001). Lysosomes are also considered important Ca^{2+} signalling platforms with a measured luminal Ca^{2+} concentration of $\sim 600 \mu\text{M}$ (Appelqvist et al., 2013; Christensen et al., 2002). Various Ca^{2+} channels reside within the lysosomal membrane including the TRP-mucolipin (TRPML) subfamily and the Two-pore channels (TPCs). Lysosomal Ca^{2+} signals have been implicated in various physiological and pathophysiological processes (LaPlante et al., 2004). The importance of lysosomal Ca^{2+} signalling for normal cellular activity can be illustrated by considering the process of macro-autophagy (MA). MA is a fundamental homeostatic pathway for the bulk degradation of cellular macromolecules in response to: 1) Nutrient stress or 2) The accumulation of unwanted or potentially harmful dysfunctional cellular components. MA is ultimately dependent upon lysosomes and specific Ca^{2+} signals initiating from lysosomes control MA by regulating calcineurin- mediated stimulation of transcription factor EB

(TFEB), a master transcriptional regulator of genes involved in MA. Activated calcineurin dephosphorylates TFEB allowing its nuclear translocation and transcriptional up regulation of MA specific genes (Medina et al., 2015). Lysosomal storage diseases are mainly linked to impaired lysosome hydrolase function, however two neurodegenerative diseases are connected directly to defective lysosomal Ca^{2+} handling, which are Niemann-Pick type C1 (NPC-1) and Mucopolysaccharidosis IV (MLIV) (Cantiello et al., 2005; LaPlante et al., 2002; Lloyd-Evans et al., 2008). NPC-1 disease is caused by a mutation in the acid compartment protein NPC-1. The main feature of this disorder is the accumulation of lipids including sphingosine, glycosphingolipids, sphingomyelin and cholesterol, however the mechanism of the lipid storage defect and how this causes a neurodegeneration remains unknown. Lloyd-Evans *et al.* demonstrated 65% reduced Ca^{2+} release from acidic compartments in NPC-1 mutant cells compared to control cells and this preceded the accumulation of lipids (Lloyd-Evans et al., 2008). In MLIV, mutations in the Ca^{2+} permeable cation channel TRPML-1 inhibits its activity. Impaired Ca^{2+} release through these channels inhibits lysosomal membrane fusion events leading to enlarged lysosomes, a typical pathological feature found in MLIV patients' cells. Considering the importance of Ca^{2+} transients during mitosis localised lysosomal Ca^{2+} signalling is an attractive potential function for these organelles during cell division.

Considering lysosomes emergent roles in various cellular processes the involvement of these highly dynamic organelles in mitosis requires further investigation. As aforementioned, lysosomes might act as Ca^{2+} signalling

platforms, as membrane donors as catabolic organelles or any combination of these functions.

Results from extensive cellular localisation analyses presented in the previous chapters of this thesis have demonstrated cell cycle dependent redistribution of lysosomes, which is lost upon CaBP7 depletion. CaBP7 depletion caused cytokinesis failure and the accumulation of binucleate cells. This phenotype could be due to the loss of lysosome redistribution during cytokinesis, implying an indispensable requirement for lysosomes during cell division. Therefore this chapter aims to reveal the functional importance of lysosomes during mitosis.

5.2. Results

5.2.1. Generation of a genetically encoded lysosomal Ca^{2+} sensor

Since lysosome Ca^{2+} transients are clearly important in various cellular processes initial studies were designed to investigate whether localised lysosomal Ca^{2+} signalling plays a role during cell division. Genetically encoded Ca^{2+} sensors are useful tools for the study of spatially restricted Ca^{2+} signals as they can be expressed within cells and uniquely targeted to specific organelles through fusion to organelle specific proteins or the targeting motifs of such proteins. GCaMPs are the simplest genetically encoded Ca^{2+} indicators (Miyawaki et al., 1997; Nakai, Ohkura, & Imoto, 2001). GCaMPs are constructed by fusing a circularly permuted green fluorescent protein (GFP) to the Ca^{2+} binding protein calmodulin (CaM) and the CaM- binding M13 peptide of myosin light chain kinase. In the presence

of Ca^{2+} , Ca^{2+} binding to CaM causes a conformational shift that permits interaction with the M13 sequence leading to increased quantum yield from the GFP (Diagram 5.1.).

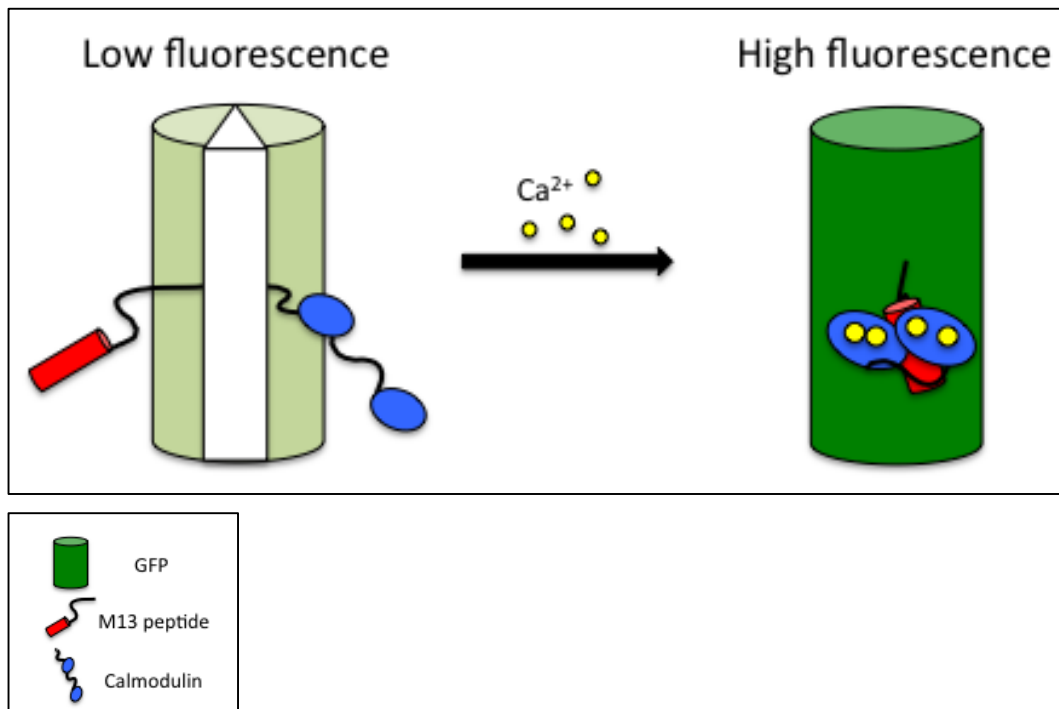


Diagram 5.1. The GCaMP Ca^{2+} sensor. The Ca^{2+} sensor GCaMP is a circularly permuted GFP where the native N- and C- termini are fused together and new termini are created from the core of the GFP to link to CaM and M13; the addition of Ca^{2+} causes CaM to bind to M13, which leads to enhanced GFP fluorescence.

Various GCaMP variants are available depending on their Ca^{2+} binding affinity, sensitivity, kinetics and response amplitude (T.-W. Chen et al., 2013). Here the GCaMP6s mutant was chosen, as it possesses a high Ca^{2+} affinity that is necessary for small Ca^{2+} transient measurements (T.-W. Chen et al., 2013). GCaMP6s was targeted to the cytoplasmic surface of lysosomes by

fusion to the most abundant lysosome membrane protein. LAMP1. LAMP1 is a single pass type I membrane protein that has a functional amino terminal luminal domain and short carboxyl terminal tail that is exposed on the lysosomal surface. Therefore, the GCaMP6s tag was appended to the carboxyl terminal tail of LAMP1 to ensure exposure of the sensor to Ca^{2+} transients in close proximity to the cytoplasmic surface of the lysosome, i.e. those emanating from the lysosome itself. Firstly, the correct expression of the untargeted GCaMP6s and LAMP1-GCaMP6s constructs was validated by western blot analysis of transfected HeLa cell lysates. The observed molecular weights of GCaMP6s and LAMP1-GCaMP6s were 50 kDa and 150 kDa, respectively (expected size 50 kDa for GCaMP6s and 150 kDa for LAMP1-GCaMP6s). The higher observed size of LAMP1-GCaMP6s can be explained by the fact that the mature form of LAMP1 is heavily glycosylated. Secondly, it was important to verify the correct localisation of LAMP1-GCaMP6s by immunofluorescence on fixed cells using a GFP specific antibody. The confocal images illustrated the expected distribution of LAMP1-GCaMP6s to punctate structures concentrated in a perinuclear region in addition to a second population of vesicles distributed throughout the cell cytoplasm (Figure 5.1. C and D). Co- transfection with LAMP1-mCherry or staining with LysoTracker® Red clearly demonstrated extensive co-localisation, proving the correct lysosomal targeting of LAMP1-GCaMP6s (Figure 5.2. A and B).

The next step was to evaluate the ability of LAMP1-GCaMP6s to detect Ca^{2+} transients at the lysosomal surface. Genetically encoded Ca^{2+} sensors are designed to have a low GFP fluorescence at resting Ca^{2+} concentrations,

which increases upon $[Ca^{2+}]$ elevation in close proximity to the sensor. During live cell imaging of LAMP1-GCaMP6s transfected cells, spontaneous fluctuations in GFP fluorescence were observed consistent with spontaneous Ca^{2+} signalling events in cultured cells. Figure 5.3. A displays still images from a representative cell at interphase exhibiting such spontaneous Ca^{2+} oscillations. Detection of spontaneous Ca^{2+} signals indicated that the LAMP1-GCaMP6s is functional as a spatially restricted Ca^{2+} probe however the precise origin of the Ca^{2+} signals in these observations could not be determined. Figure 5.3. B shows the monitored fluorescence fluctuations.

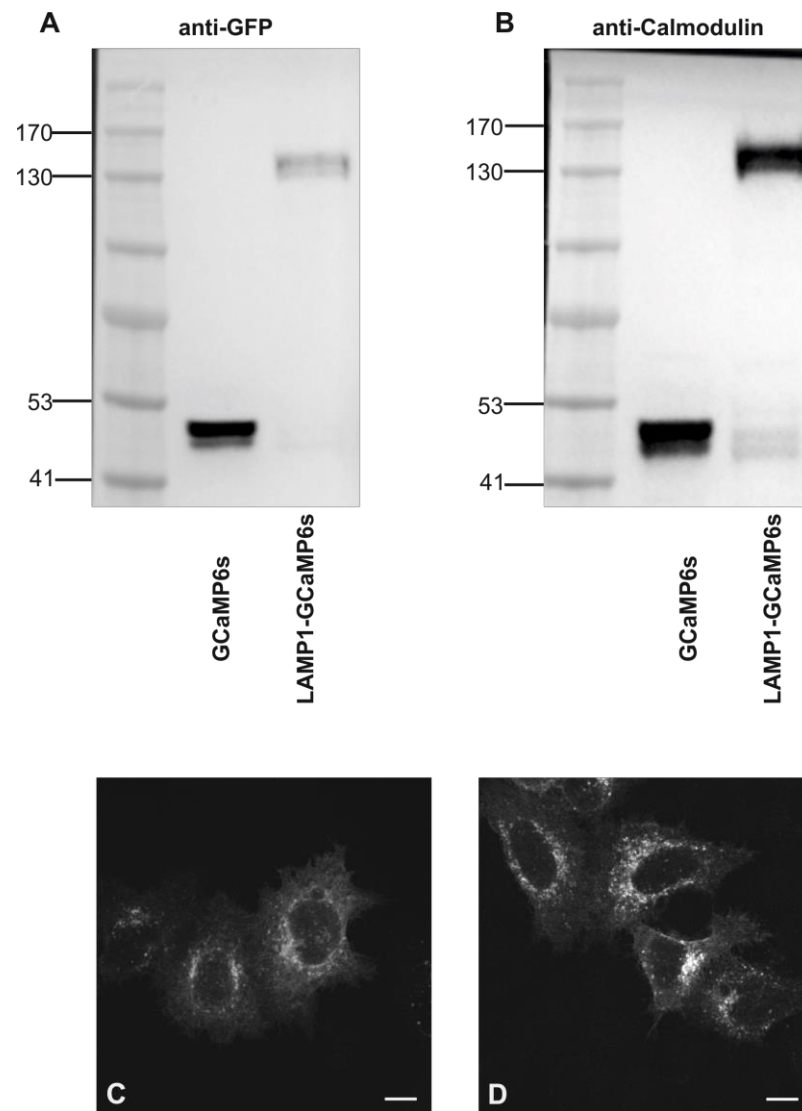


Figure 5.1. Expression of LAMP1-GCaMP6s in HeLa cells. HeLa cells expressing LAMP1-GCaMP6s were analysed by western blotting and immunofluorescence. Western blot detecting GCaMP6s using A) anti-GFP, B) anti-Calmodulin. Position of molecular weight standards (kDa) are shown to the left of each blot; C and D) Representative images of LAMP1-GCaMP6s expressing HeLa cells immunostained with anti-GFP antibody. Scale bars = 10 μm .

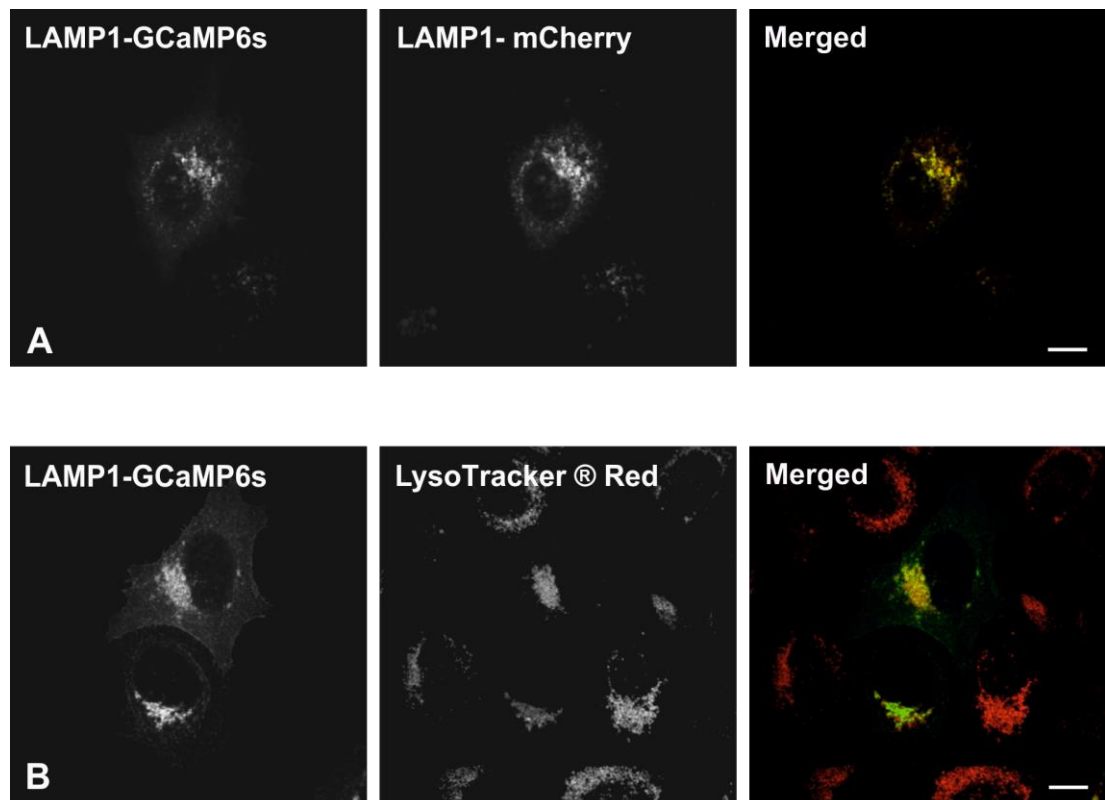


Figure 5.2. Localisation study of LAMP1-GCaMP6s. LAMP1-GCaMP6s expressing HeLa cells were co-transfected or stained with lysosome markers. A) HeLa cells expressing LAMP1-GCaMP6s (green) and LAMP1-mCherry (red), B) LAMP1-GCaMP6 (green) expressing cells were stained with LysoTracker ® (red), co-localisation appears yellow in the overlay images. Scale bars = 10 μ m.

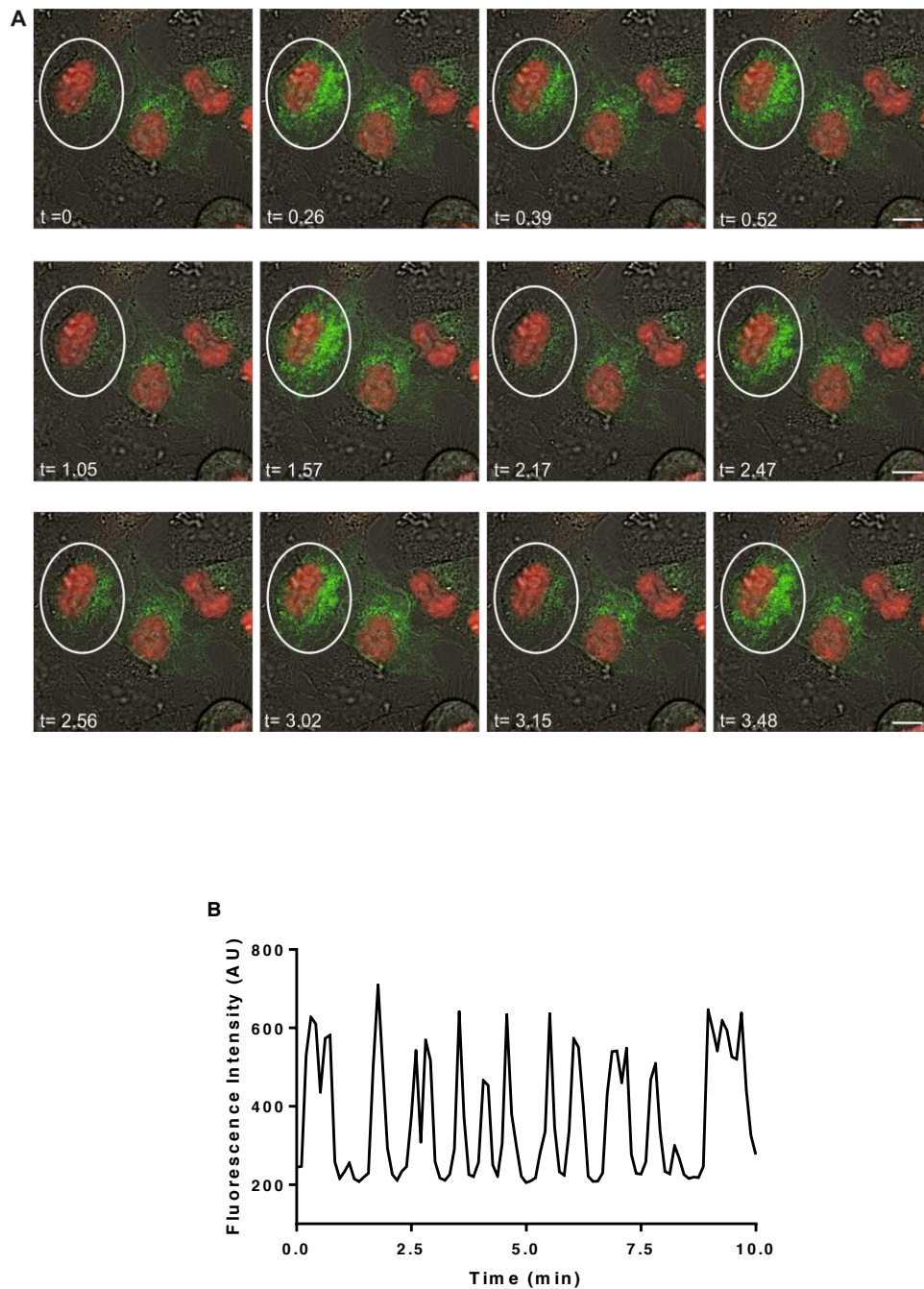


Figure 5.3. Spontaneous Ca^{2+} signalling in HeLa cells. Live HeLa cells expressing H2B-mCherry (red) and LAMP1-GCaMP6s (green) were imaged. A) Stills from time-lapse imaging, white ellipse highlights a cell which exhibited spontaneous Ca^{2+} signalling activity. Acquisition frame rate (F) = 1 Frame/6.5 sec. Time (t) is shown in minutes. Scale bar = 10 μm . B) Fluorescence intensity (arbitrary units (AU)) plotted against time (minutes) in the ROI bounded by the white ellipse defined in (A).

In order to illustrate that the Ca^{2+} signal sensed by the LAMP1-GCaMP6s is specific to lysosomes, cells expressing LAMP1-GCaMP6s were treated with histamine an extracellular agonist, acting through its H1 receptor, capable of releasing Ca^{2+} from intracellular compartments (Diarra, Wang, Garneau, Gallo-Payet, & Sauvé, 1994; Esposito et al., 2011; Haynes, Tepikin, & Burgoyne, 2004). Histamine has been shown to release Ca^{2+} similar to nicotinic acid-adenine dinucleotide phosphate (NAADP) generated Ca^{2+} , namely from non-ER stores (Churchill et al., 2002; Diarra et al., 1994). NAADP has been shown to mobilise lysosomal Ca^{2+} through the lysosomal Ca^{2+} channel TPC1 (Brailoiu et al., 2009). Therefore histamine was applied in this experiment to evoke non-ER calcium release, which could then be from the lysosomal stores to test this construct. Histamine evoked a robust Ca^{2+} response to an antagonist known to generate NAADP, which is detected by the GCaMP6s (Figure 5.4. C). Figure 5.4. A and B show the cell pre- and post- histamine treatment, clearly displaying an increase in $[\text{Ca}^{2+}]$, evaluated by the measurable increase in GFP fluorescence intensity. Since the calcium signal trace doesn't show a typical CICR as typically seen upon ER calcium release, it can be concluded that this calcium release is most likely from a non-ER store, namely lysosomes.

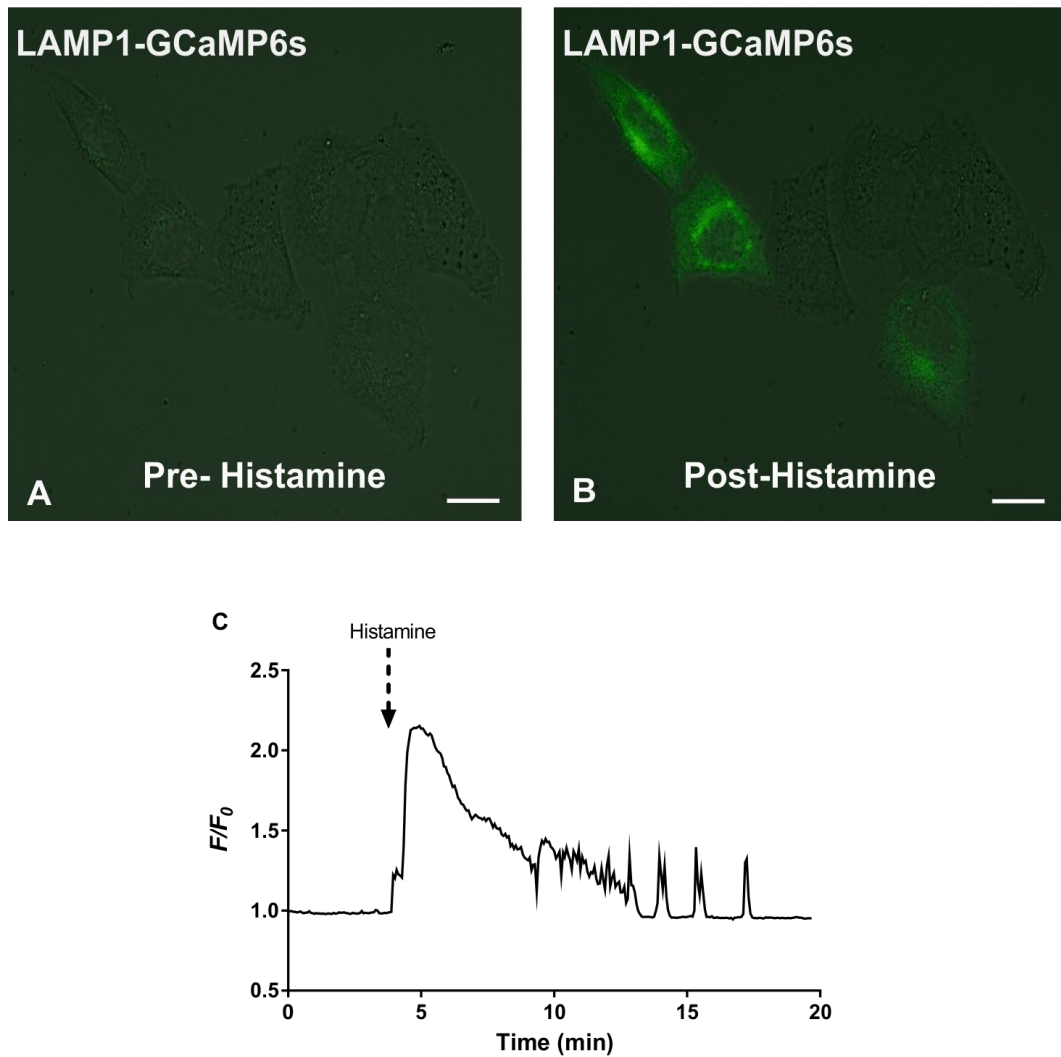


Figure 5.4. LAMP1-GCaMP6s functionality test with Histamine. HeLa cells expressing LAMP1-GCaMP6s were imaged live. A) Still from the time lapse imaging pre-histamine treatment, B) Still of the same cells post-histamine treatment, Scale bars = 10 μm ; C) Fluorescence intensity analysed as the ratio F/F_0 (Fluorescence intensity (AU) at time (t)/Fluorescence intensity (AU) at time=0). Acquisition frame rate (F) = 1 frame/4 seconds. Histamine was applied after 4 minutes of imaging as indicated with the arrow.

The ultimate goal was to employ this targeted and functionally verified Ca^{2+} sensor in actively dividing HeLa cells. By monitoring the GFP fluorescence of cells expressing LAMP1-GCaMP6s during cell cycle progression, new insights into lysosomal Ca^{2+} signalling could potentially be obtained.

HeLa cells were co-transfected with Histone 2B (H2B) - mCherry and LAMP1-GCaMP6s. H2B, a DNA marker, fulfilled a dual role to firstly allow identification of transfected cells, since GCaMP6s fluorescence is low (often invisible) at resting cytoplasmic $[\text{Ca}^{2+}]$, and secondly to permit accurate monitoring of cell cycle progression. Figure 5.5. A displays representative still images from a live cell imaging experiment, where the cell undergoes successful mitosis and completes cytokinesis. The GFP fluorescence remains constant throughout the cell cycle stages. The fluorescence intensity analysis revealed no rise in GFP fluorescence indicating no detectable lysosomal Ca^{2+} signalling (Figure 5.5. C). Since no fluorescence increase was observed, to verify that the imaged cell was in fact expressing LAMP1-GCaMP6s, Ionomycin was applied post cell division. Ionomycin is a Ca^{2+} ionophore, which permits extracellular Ca^{2+} to enter the cell cytoplasm. Figure 5.5. C shows an image of the tracked cell and highlights a robust GFP fluorescence rise post- Ionomycin treatment, indicating LAMP1-GCaMP6s expression. This experiment was repeated (n=15) without detecting a Ca^{2+} signal. Figure 5.5. illustrates data from one representative experiment.

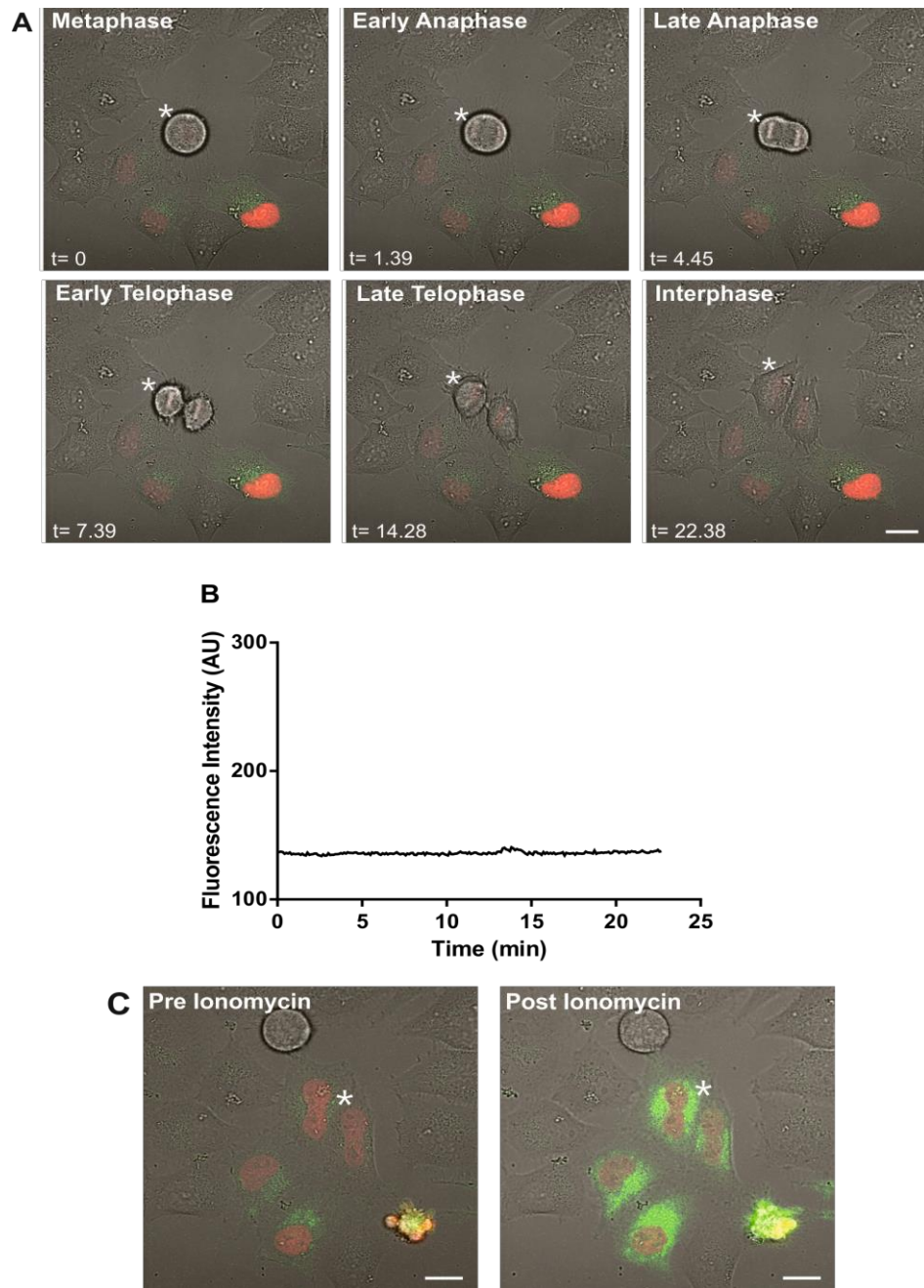


Figure 5.5. Lysosomal Ca^{2+} signalling during cell division. HeLa cells expressing H2B-mCherry and LAMP1-GCaMP6s were imaged during mitosis. A) Representative still images from time-lapse imaging of LAMP1-GCaMP6s (green) and H2B-mCherry (red) expressing cells. Acquisition frame rate (F) = 1 Frame/6.2 seconds. Time (t) is shown in minutes. B) Fluorescence intensity analysis of the time lapse imaging. C) Image of the same cells shown in A after post-ionomycin treatment at the end of the experiment to verify expression of LAMP1-GCaMP6s (green). Scale bars = 10 μm

5.2.2. Investigating alternative lysosomal functions during mitosis

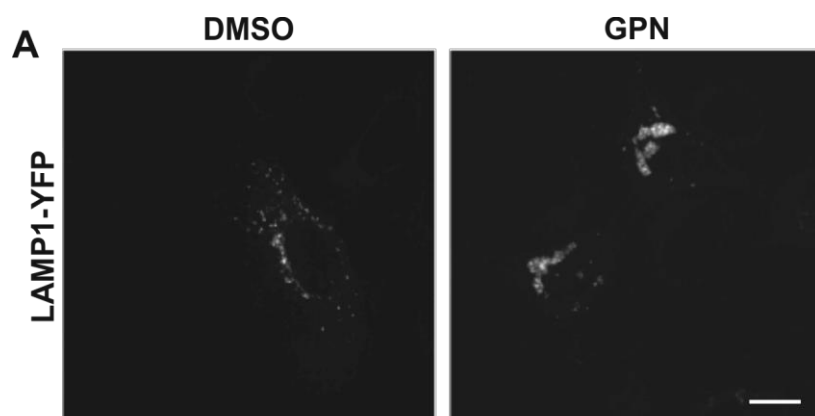
Since lysosomal Ca^{2+} signals were undetectable with the LAMP1-GCaMPs probe during cell division other potential mitosis specific functions of lysosomes were next investigated. This was a valid extension to these studies in light of data from Chapter 4 of this thesis showing that loss of lysosome clustering elicited cytokinesis failure and increased binucleate cell frequency implying correct lysosome positioning/spatially restricted activity is required for normal mitotic progression.

To further dissect lysosomal function during cell division, cells were treated with compounds known to inhibit specific lysosome functions to evaluate whether any of them influenced cell division.

To directly test whether lysosomes are required during cell division, lysosomes were chemically disrupted and cell cycle progression monitored. For this purpose glycyl- L- phenylalanine 2-naphthylamide (GPN) was applied to cells, which specifically disrupts lysosomes. GPN is exclusively hydrolysed by the lysosome resident enzyme cathepsin C, which in turn causes osmotic swelling, perforation and eventual lysis of lysosomes (Berg, Strømhaug, Løvda, Seglen, & Berg, 1994). LAMP1-YFP expressing cells treated with GPN were fixed and imaged (Figure 5.6. A). GPN treatment resulted in extensive lysosomal swelling and morphological changes compared to controls. Next, cells loaded with LysoTracker® were treated with GPN and imaged overnight. LysoTracker® is a fluorophore linked to a weak base only partially protonated at neutral pH, therefore selectively accumulated into acidic organelles. As expected, GPN treatment caused loss of the LysoTracker® fluorophore due to lysosome perforation and collapse of the

pH gradient across the lysosome membrane. Importantly, dividing cells treated with GPN exhibited cytokinesis failure and coalesced back into a single binucleate cell (Figure 5.6.). These results are reminiscent of manipulations that prevent normal lysosomal clustering at mitosis (Chapter 4).

In order to quantify these observations, cells that started to divide were counted as “dividing cells” and cells which failed to complete mitosis, were counted as “non-dividing cells”. Approximately 24% of the cells did not divide successfully upon GPN application (Figure 5.10.), similar to the results obtained upon CaBP7 depletion. GPN caused a 10 times higher mitotic failure rate compared to the vehicle treated cells (2.5%). These results support some aspect of lysosome activity as important during mitosis.



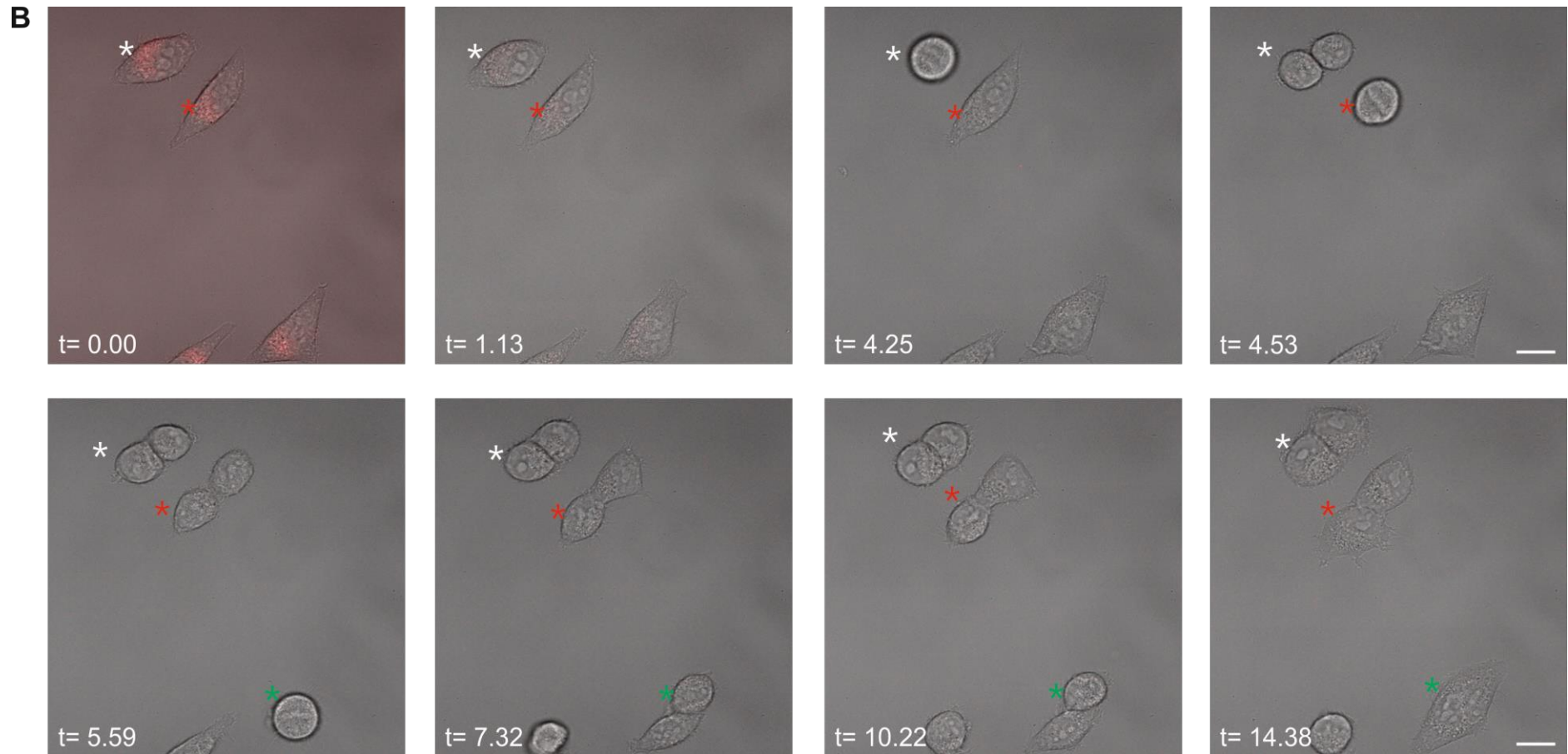
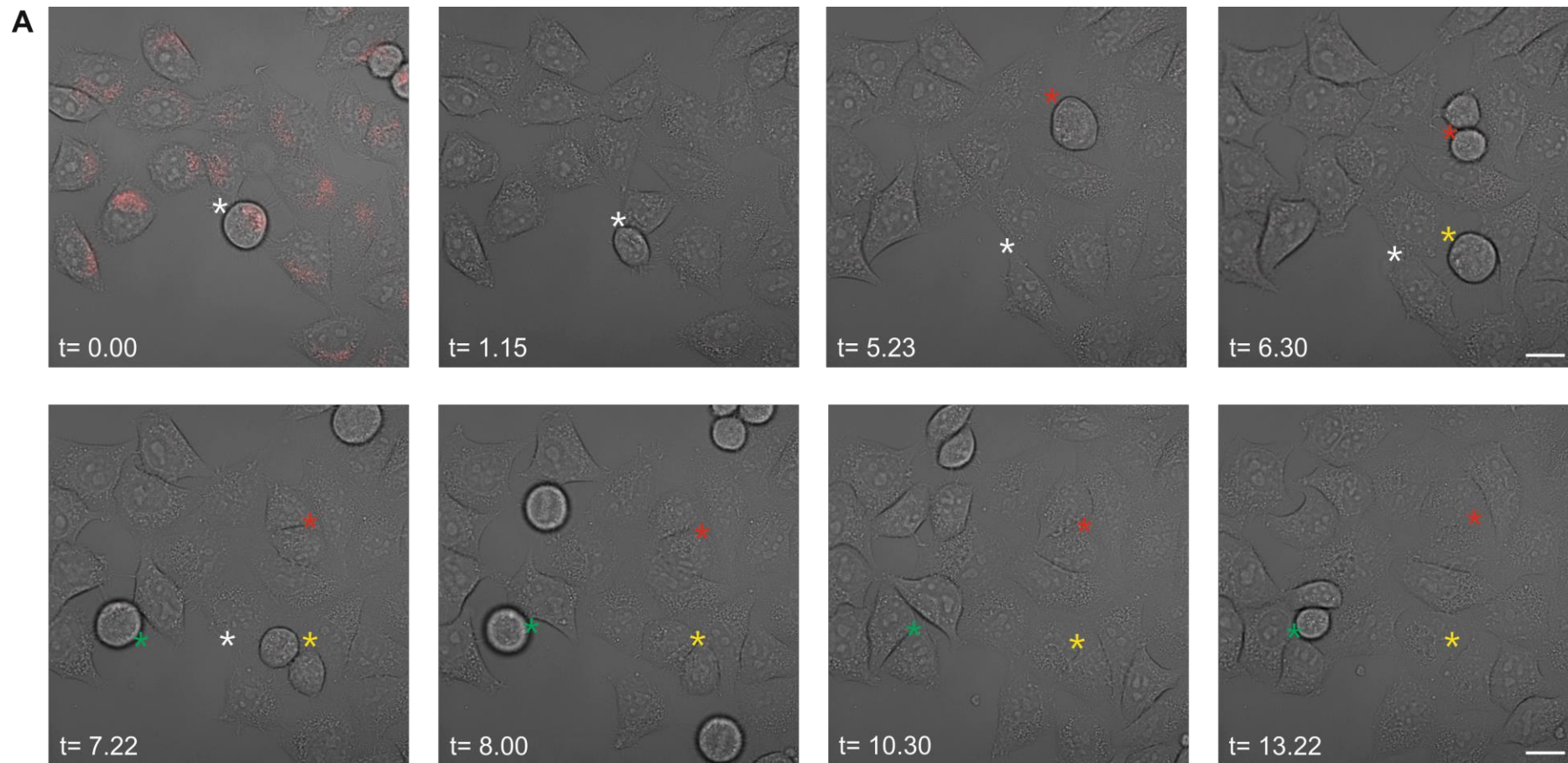


Figure 5.6. Disruption of lysosomes during cell division. Effect of GPN on fixed and live HeLa cells. A) LAMP1-YFP expressing cell were treated with DMSO (vehicle control) or 50 μ M GPN for 2 hours prior to fixation. B) HeLa cells loaded with LysoTracker® Red were treated with 50 μ M GPN and imaged overnight. Acquisition frame rate (1F) = 1 frame/ 7.32 minutes. Time (t) shown in hours. Asterisks in various colours indicate dividing cell. Scale bars = 10 μ m.

To investigate specific lysosome functions that might be implicated in cell division, bafilomycin A1 and folimycin were utilised. Bafilomycin A1 is a specific vacuolar ATPase (V-ATPase) inhibitor (Bowman, Siebers, & Altendorf, 1988; Dröse et al., 1993) and has been shown to inhibit autophagy by specifically preventing the fusion of autophagosomes with lysosomes (Yamamoto et al., 1998). Folimycin also known as concanamycin is a more recently established drug which acts in the same manner as bafilomycin, but which is a considerably more potent drug (Dröse et al., 1993). By obstructing the V-ATPase the pH gradient in the lysosomes is ablated and the majority of the luminal hydrolases, which require the acidic pH to function, lose activity (Saftig & Klumperman, 2009). These agents therefore simultaneously inhibit two aspects of lysosome function, fusogenic potential and proteolytic activity. HeLa cells loaded with LysoTracker® were treated with bafilomycin or folimycin and monitored overnight to track dividing cells. Since both drugs alkalise lysosomal pH the LysoTracker® fluorophore was lost into the cytoplasm (Figure 5.7.). However bafilomycin and folimycin treated cells successfully divided and completed cytokinesis (Figure 5.7 A, B). Figure 5.10. summarises the quantification of these analyses, highlighting no effect on mitosis upon V-ATPase inhibition.



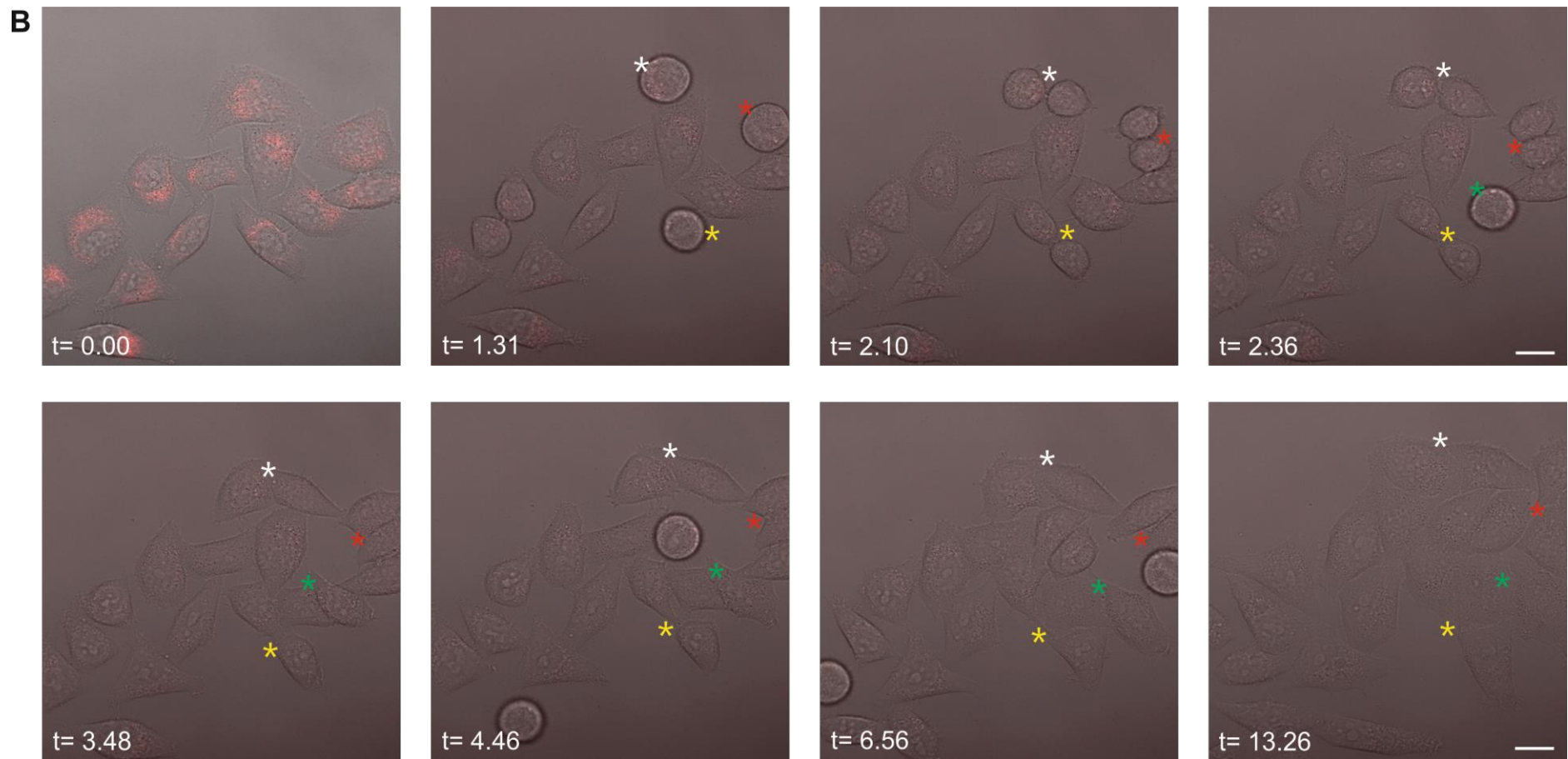


Figure 5.7. Inhibition of V-ATPase activity during cell division. HeLa cells loaded with LysoTracker ® Red were treated with A) Bafilomycin (Frame rate 1F = 7.50 minutes) and B) Folimycin (Frame rate 1F= 6.50 minutes) and imaged overnight. Time (t) is shown in hours. Asterisks in various colours indicate dividing cells. Scale bars = 10 µm.

Next, pepstatin A was utilised to specifically assay the importance of lysosomal proteolytic functions during mitosis. Pepstatin A specifically inhibits cathepsin D function, the main enzyme involved in lysosomal catabolism (Benes, Vetvicka, & Fusek, 2008; Kominami, Ueno, Muno, & Katunuma, 1991). HeLa cells loaded with LysoTracker® were treated with pepstatin A and monitored overnight to track dividing cells (Figure 5.8). Lysosomal LysoTracker® fluorescence persisted during these experiments, as expected, however the signal intensity decreased because of photo bleaching (Pierzyńska-Mach, Janowski, & Dobrucki, 2014). Nevertheless, a dim signal was observable until the end of the imaging (as long as 17 hours). Quantification of successfully dividing cells demonstrated no effect of pepstatin A on cell division (Figure 5.10.), hence lysosome digestion appears redundant for successful mitosis however important control experiments testing cathepsin D inhibition need to be conducted before this can be confirmed.

In an extension to these analyses, the compound vacuolin-1, a cell permeable triazine, was employed to specifically inhibit lysosome fusion with the plasma membrane (Cerny et al., 2004). The application of vacuolin-1 elicited the most dramatic effect on cell division. Figure 5.9. depicts images from a live cell experiment, demonstrating the morphological changes and eventual mitosis failure in vacuolin-1 treated cells where ~42% of cells failed to complete mitosis (Figure 5.10.). These results suggest that lysosome exocytosis could be an important and required function during cell division.

Vacuolin-1 has been shown to impair lysosome fusion although the exact mechanism is unknown. Recently, it was shown that vacuolin-1 has also an impact on early endosomes (J. Yue & Lu, 2014).

In order to distinguish whether the observed morphological changes are specific to lysosomes or possibly to early endosomes, cells expressing Rab5a- YFP and LAMP1-YFP were treated with vacuolin-1, fixed and imaged. Representative images illustrate a striking change in the lysosomes (LAMP1-YFP) (Figure 5.11. A), whereas no obvious changes are visible in early endosomes (Rab5a-YFP) (Figure 5.11. B). Additionally, particle count analysis after vacuolin-1 showed a significant reduction of particles due to morphology changes for LAMP1, while no differences in the particle count between Rab5a vacuolin-1 treated and non-treated cells was observed (Figure 5.11. C). This assay verified vacuolin-1 treatment to only induce morphological changes of lysosomes and not to influence endosomes. Therefore the dramatic effects of vacuolin-1 treatment on cell division are most likely due to specific disruption of lysosome function.

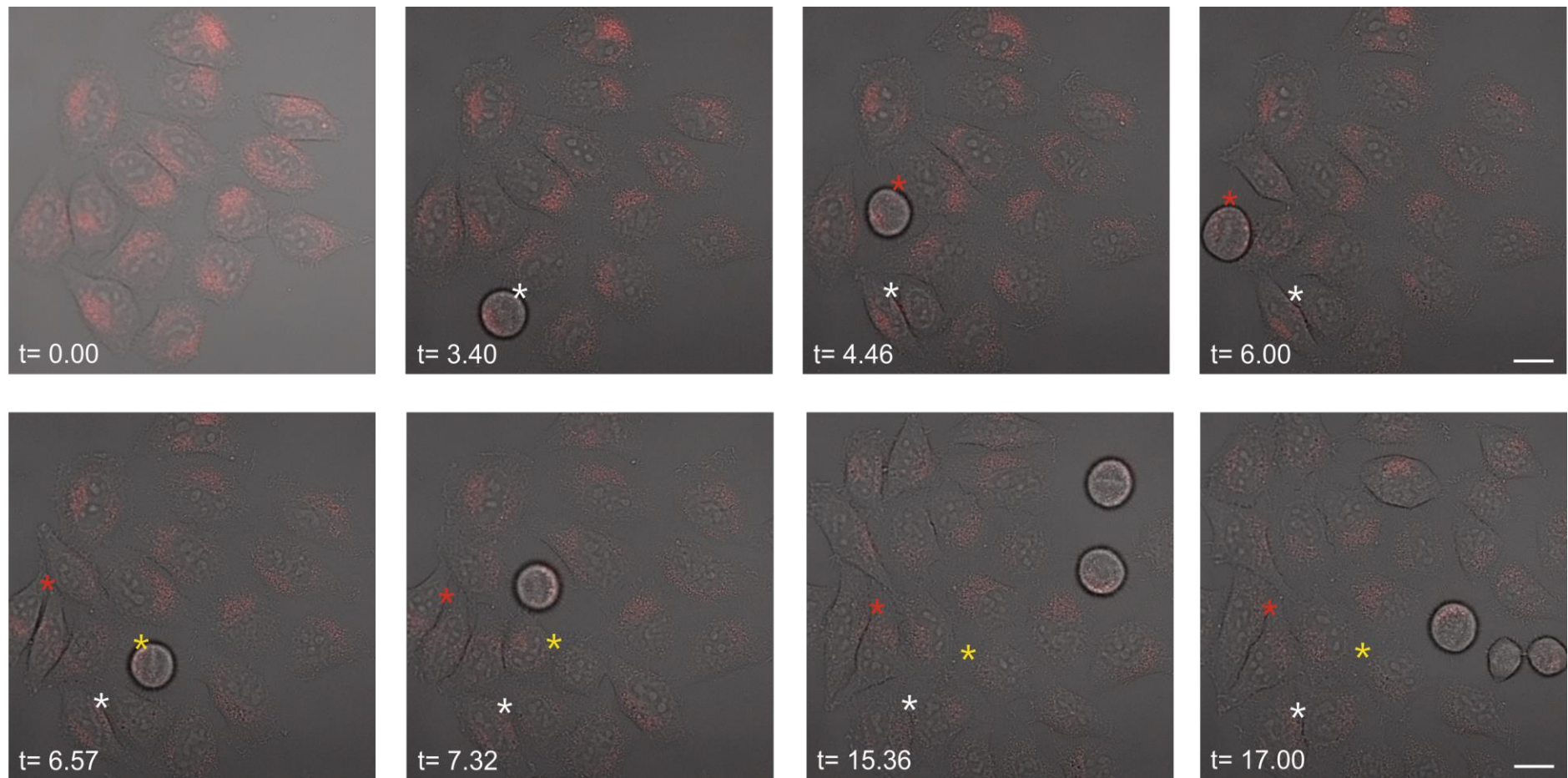


Figure 5.8. Disruption of lysosome catabolism function during cell division. HeLa cells loaded with LysoTracker® Red were treated with pepstatin A and imaged overnight. Acquisition frame rate (1F) = 7.32 minutes. Time (t) is shown in hours. Asterisks in various colours indicate dividing cell. Scale Bars = 10 μ m.

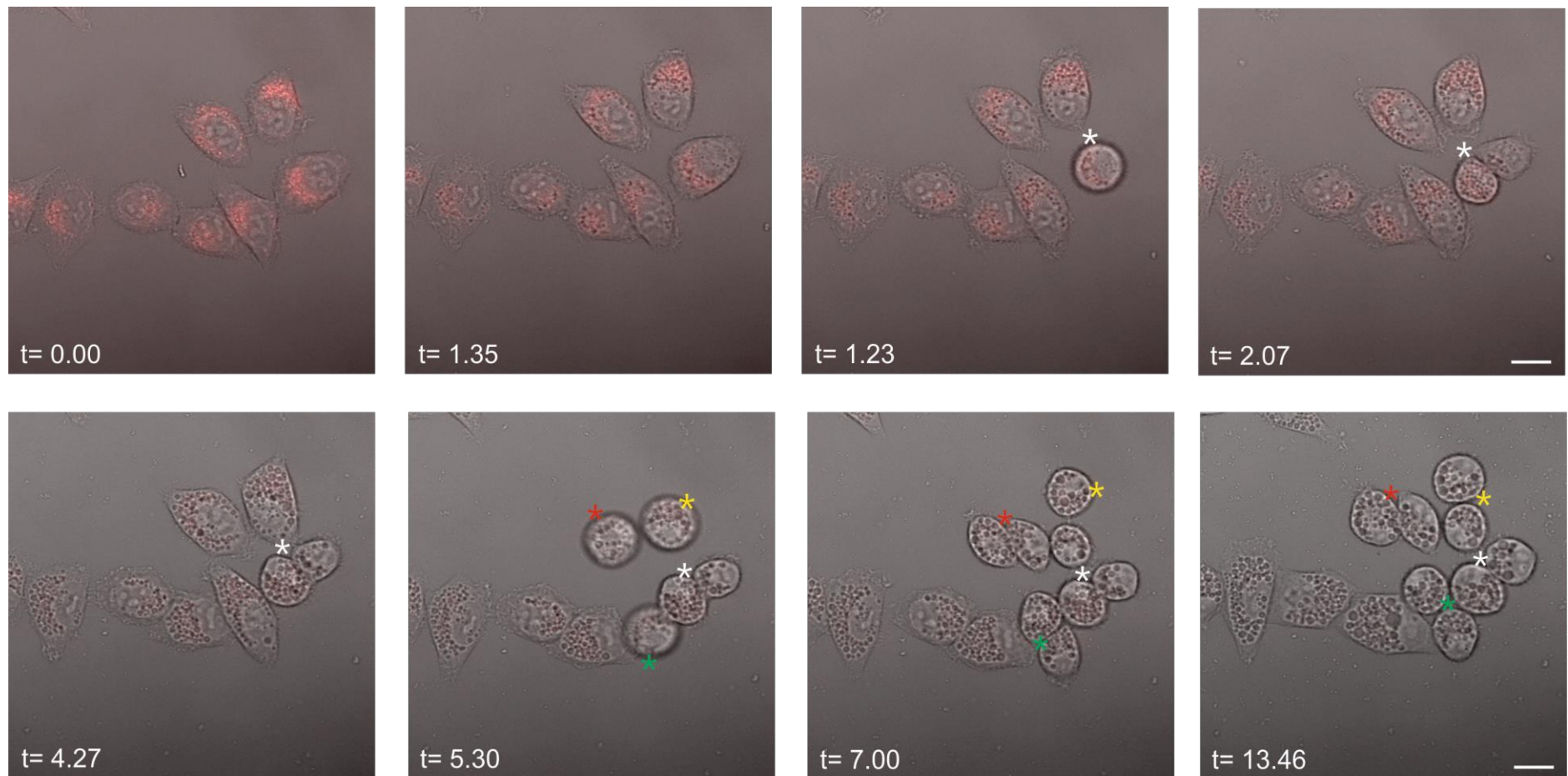


Figure 5.9. Disruption of lysosomal exocytosis during cell division. HeLa cells loaded with LysoTracker® Red were treated with vacuolin-1 and imaged overnight. Acquisition frame rate (1F) = 6.35 minutes. Time (t) is shown in hours. Asterisks in various colours indicate dividing cell. Scale bars = 10 μ m.

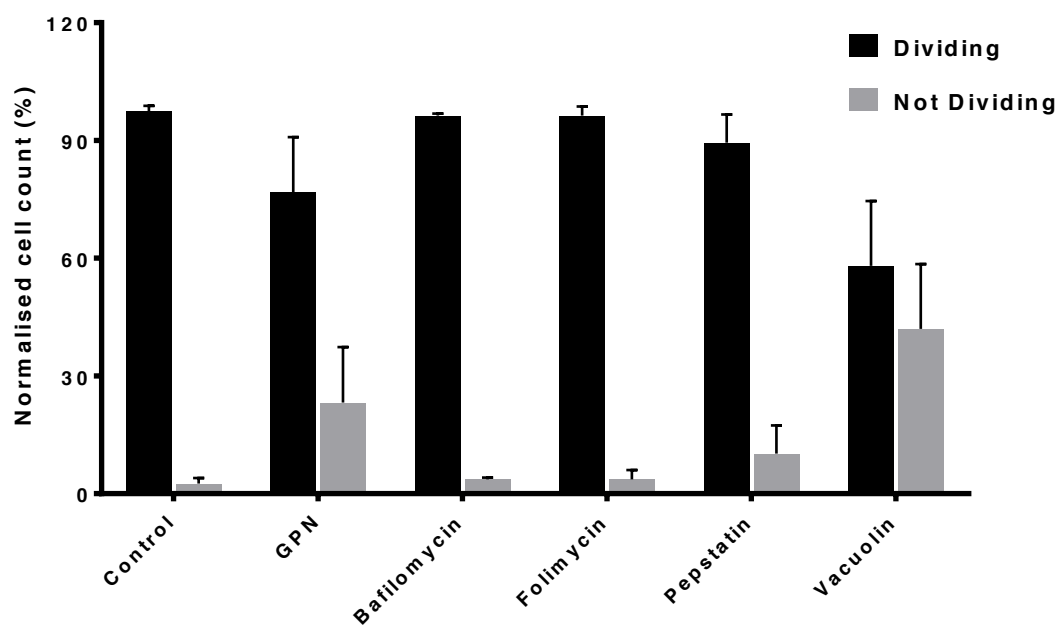


Figure 5.10. Quantification of drug treatment effects on cell division in HeLa cells. All data are plotted as average \pm SEM (n=3 independent experiments). Vehicle control was DMSO.

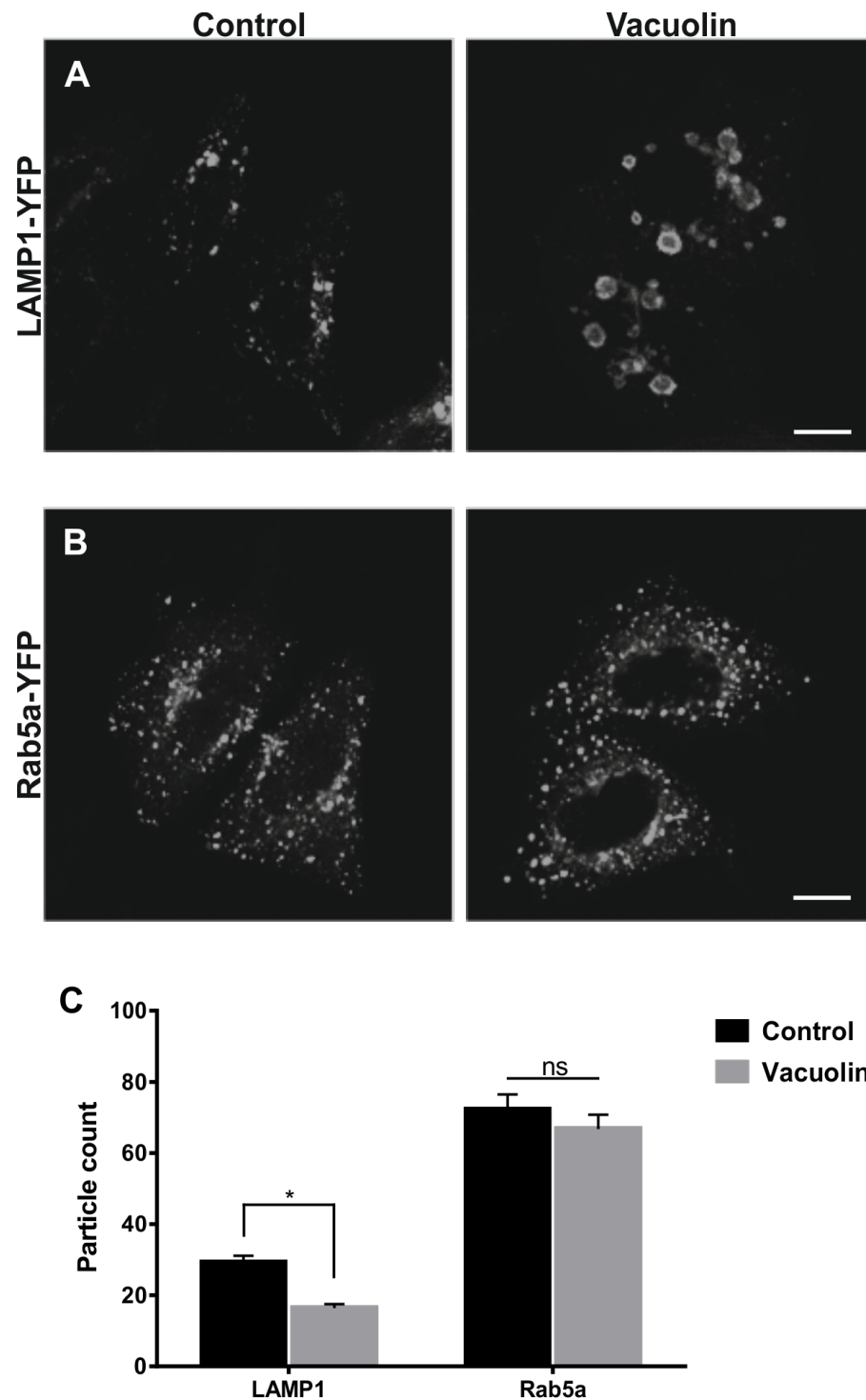


Figure 5.11. Vacuolin-1 effects on early endosomes and lysosomes. Representative images of vacuolin-1 treatment on A) LAMP1-YFP expressing and B) Rab5a-YFP expressing cells. Scale bars = 10 μ m. C) Quantification of number of particles from A and B. Averaged data plotted as \pm SEM and analysed for statistical significance using coupled ANOVA one-way analysis (* $p < 0.05$).

5.3. Discussion

Cell division is a complex process that requires highly organised spatio-temporal regulation. It has been shown that phosphorylation (primarily by Cdks) plays a crucial role during cell division, however relatively little is known about the importance of Ca^{2+} signalling. In this thesis I demonstrated that CaBP7 down regulation led to an enhanced frequency of binucleate cells, suggesting that CaBP7 is important in the later stages of mitosis, namely in telophase or cytokinesis. Interestingly CaBP7 adopted a telophase specific clustering distribution to either side of the cell nuclei, along with lysosomes, suggesting that lysosome localisation is also critical during telophase. Importantly, the key observation was that in the CaBP7 knockdown cells, the distinctive telophase clustering phenomena of lysosomes was lost. These findings propose not only that CaBP7 influences lysosome trafficking during cell division, but also that the down regulation of CaBP7 and subsequent increase in binucleate cell numbers might be connected to lysosome mislocalisation during mitosis. Collectively these results suggest that lysosomes might play a fundamental role in successful completion of mitosis. Therefore the work in this chapter aimed to link established lysosome functions to mitosis in mammalian cells.

Lysosomes are now accepted as Ca^{2+} signalling organelles and known to play a key role in cellular Ca^{2+} homeostasis (Appelqvist et al., 2013; Christensen et al., 2002). Studies have revealed the importance of lysosomal Ca^{2+} signalling for many physiological functions (A. J. Morgan et al., 2011). Lysosomal Ca^{2+} is required to modulate lysosomal luminal pH, which in turn is essential to normal lysosome function (Christensen et al., 2002). In

addition to ensuring normal lysosome physiology, lysosomal Ca^{2+} also fulfils specific signalling roles. Ca^{2+} released from these organelles can act as a 'trigger' for Ca^{2+} induced Ca^{2+} release (CICR) from the ER. This phenomena has been characterised in gastric and arterial smooth muscle cells where lysosomal Ca^{2+} release has been shown to elicit global Ca^{2+} signals (Boittin, Galione, & Evans, 2002; Pereira et al., 2014). Lysosomal Ca^{2+} release also appears important during apoptosis where it is involved in externalization of phosphatidylserine (PS) to the cell surface. The appearance of PS on the outer leaflet of the plasma membrane is a key signal during apoptosis for eventual removal of the apoptotic cell (Mirnikjoo, Balasubramanian, & Schroit, 2009). Furthermore it has been shown that lysosomal Ca^{2+} misregulation can result in serious lysosomal storage diseases (Lloyd-Evans et al., 2008). Collectively, these studies demonstrate an emerging role for lysosomes as Ca^{2+} signalling platforms in health and disease. The role of lysosomes during cell division has been overlooked and, in view of the recent advances in our knowledge regarding lysosome functions in mammalian cells as discussed above, their potential influence on this process merits further investigation. Ca^{2+} oscillations have been measured during mitosis in non-mammalian cells, however the exact role and functionality of mitosis specific Ca^{2+} signalling remains unknown (Ciapa, Pesando, Wilding, & Whitaker, 1994; Whitaker, 1997; 2008). Since depletion of CaBP7, a Ca^{2+} binding protein, led to lysosome mislocalisation one potential hypothesis is that lysosomes act as Ca^{2+} signalling platforms during mitosis. To investigate this idea a novel lysosomal Ca^{2+} sensing probe was engineered. By fusing the genetically encoded Ca^{2+} sensor (GCaMP6s) to the lysosome associated-

membrane protein 1 (LAMP1), it was possible to measure Ca^{2+} oscillations in close proximity to (and possibly originating from) lysosomes. LAMP1-GCaMP6s localised to lysosomes as identified by co-expression with a second LAMP1 construct (LAMP1-mCherry) or staining with LysoTracker®. Upon confirmation of correct localisation, the Ca^{2+} sensing functionality of LAMP1-GCaMP6s was verified. LAMP1-GCaMP6s expressing cells were treated with histamine to mobilise Ca^{2+} from intracellular compartments, potentially including lysosomal stores (Esposito et al., 2011). As expected, a GFP fluorescence increase was observed upon histamine treatment, indicating an increase in $[\text{Ca}^{2+}]$ at the cytoplasmic surface of the lysosomes and proving the capability of lysosomally targeted GCaMP6s to sense Ca^{2+} signals (Figure 5.4.). Subsequently, cells expressing the LAMP1-GCaMP6s construct were monitored throughout cell division however no detectable fluorescence changes could be observed during progression of the cell cycle (Figure 5.5.). These results led to the conclusion that lysosomal Ca^{2+} signalling does not occur during mitosis. However technical or instrumental limitations might have prevented the measurement of lysosomal Ca^{2+} transients in these preliminary analyses. Various GCaMP6 mutants, with different Ca^{2+} binding properties (affinity, on/off kinetics and dynamic range), are available (T.-W. Chen et al., 2013). Three different GCaMP6 variants based on their off kinetics (slow (s), medium (m) and fast (f)) have been engineered. In this chapter GCaMP6s was employed, this version has slow on/off kinetics but is brighter and has a high Ca^{2+} affinity (144 ± 4 nM), which allows the detection of small changes in $[\text{Ca}^{2+}]$ above resting concentrations. GCaMP6f on the other hand has 6x faster on-kinetics and a reduced affinity

for Ca^{2+} (375 ± 14 nM) (T.-W. Chen et al., 2013). If mitotic Ca^{2+} transients linked to lysosomal Ca^{2+} were so fast so as to be undetectable with GCaMP6s, then GCaMP6f represents a second probe that could be utilised to further investigate lysosomal Ca^{2+} signalling in future work.

Probe sensitivity is one issue to consider when undertaking sensitive Ca^{2+} imaging experiments. A second important consideration is possible limitations inherent in the hardware being used to acquire the experimental data. Ca^{2+} signals can be temporally restricted and short lived, lasting for only tens of milliseconds. Image acquisition rate of the hardware could therefore pose a limiting factor on the detection of fleeting signalling events. Microscope acquisition speed depends on several factors, but is mainly dictated by the mechanical operation of the microscope. Here a laser-scanning confocal microscope (LSM) with a photo-multiplier tube (PMT) was used, which permitted a fastest acquisition rate of 4 seconds / frame whilst maintaining sufficient spatial resolution and image quality. PMTs have a typical detection quantum efficiency around 25% of the fluorescence signal therefore increasing scanning speeds will reduce the quality of images and introduce a further risk of photo bleaching. LSMs utilise fast axis scan mirrors /galvanometer mirrors which further limit acquisition speed. In contrast spinning disk microscopes coupled with cameras such as sCMOS or EMCCD allow collection of high-resolution images with improved acquisition rates. Additionally, resonant scanning mirrors overcome not only many speed restriction issues but also minimise photo bleaching.

Consequently even though, in the work presented in this thesis chapter, no lysosomal Ca^{2+} signals could be observed this does not disprove the

existence of such signals. The probability that the signal was missed due to instrumental and technical limitations exists. Therefore more advanced studies employing other GCaMPs in combination with different microscopes and settings are required to further establish whether lysosomal Ca^{2+} signalling is a genuine physiological phenomenon that is important during mammalian cell mitosis.

In order to investigate other potential functions of lysosomes during cell division in mammalian cells a further set of experiments was designed which examined how pharmacological inhibitors of lysosomal processes influenced mitosis/cytokinesis progression.

Firstly, the compound glycyl-L-phenylalanine 2-naphthylamide (GPN) was utilised to identify whether lysosomes are essential during cell division. GPN is activated upon cleavage by the lysosome resident hydrolase cathepsin C, which in turn causes osmotic perforation and eventual lysosome destruction (Berg et al., 1994; Jadot, Colmant, Wattiaux-De Coninck, & Wattiaux, 1984). GPN treatment induced mitosis failure in approximately 25% of the analysed cells, similar to CaBP7 knockdown (Figure 5.6. and 5.10.), suggesting that lysosomes are important in cell division. GPN inhibits multiple aspects of lysosome function and therefore it is not possible to determine precisely what lysosome activity is required for normal mitosis progression from these analyses. To more selectively address the role of lysosomes during mitosis more specific pharmacological agents were employed.

To assess lysosome proteolytic activity during mitosis the effects of bafilomycin/folimycin and pepstatin A were studied. Bafilomycin and folimycin have been established to inhibit specifically the proton (H^+) pumping vacuolar

– ATPase (V-ATPase) (Bowman et al., 1988; Yamamoto et al., 1998). The V-ATPase utilises the free energy of ATP hydrolysis to drive protons against their electrochemical gradient and into the lysosomal lumen (Moriyama, Takano, & Ohkuma, 1982). Acidification of the lysosome lumen is imperative since lysosomal enzymes (acid hydrolases) are only functional at an acidic pH (Eskelinen et al., 2003; Saftig & Klumperman, 2009). Therefore by applying drugs, which inhibit V-ATPase H^+ pumping collapses the pH gradient (ΔpH) across the lysosome membrane and this in turn will inhibit acid hydrolase activity, and the degradative capacity of lysosomes. The results presented in this chapter clearly demonstrate that bafilomycin and folimycin do not inhibit cell division at concentrations known to disrupt lysosomal ΔpH . Almost all cells (96%- with either drug treatment) divided normally (Figure 5.7. and 5.10.), similar to the vehicle control condition. Since disruption of the lysosome ΔpH will influence other aspects of lysosome activity proteolytic functions during mitosis were probed more directly with the protease inhibitor pepstatin A. Pepstatin A is an aspartyl protease inhibitor that inhibits cathepsin D, the main lysosomal hydrolase (Umezawa, Aoyagi, Morishima, Matsuzaki, & Hamada, 1970). Cathepsin D based on its cleavage functions has been implicated in various degradation pathways as well as in regulating apoptosis (Benes et al., 2008; Roberg & Ollinger, 1998; Roberg, Johansson, & Ollinger, 1999). By inhibiting this acid hydrolase, lysosomal protein catabolism during cell division was investigated. The application of pepstatin A did not influence mitosis (Figure 5.8. and 5.10.). Hence lysosomal digestive function, as suggested with bafilomycin and folimycin treatments, was deemed unnecessary during mitosis.

Bafilomycin and folimycin have also been shown to inhibit the process of macro-autophagy (Klionsky, Elazar, Seglen, & Rubinsztein, 2008). Controversy remains regarding a potential role for autophagy during cell division. Some studies suggest that autophagy is shut down during mitosis to protect the fragile mitotic spindle and the dividing chromosomes from off-target degradation (Eskelinen et al., 2002). Additionally the Golgi is known to fragment during mitosis and it has been speculated that in this dispersed state this organelle could be prone to destruction if autophagy were active (Furuya et al., 2010). There are contradictory studies including that of Liu *et al.* which demonstrate that autophagy is active and functions to clear defective mitochondria during mitosis via a mechanism known as mitophagy (Liu, Xie, Nguyen, Ye, & McKeehan, 2009). However in this study questions persist regarding how the temporally foreign material (chromosome and mitotic spindle) can escape and are protected from autophagy. In this chapter by employing bafilomycin/folimycin to prevent autophagosome-lysosome fusion, and thereby inhibiting macroautophagy, it would appear that the activity of this cellular process is not required for normal mitosis and completion of cell division.

Finally, lysosomal exocytosis and membrane donation during mitosis was explored. Ca^{2+} dependent fusion of lysosomes to the cell membrane by exocytosis is required to repair cell membrane wounds (Idone et al., 2008; A. Reddy et al., 2001) and vacuolin-1, a cell permeable triazine, has been shown to inhibit this process (Cerny et al., 2004). This chemical was utilised to identify whether a membrane donation function of lysosomes is required during cell division. A profound effect of vacuolin-1 on cell division was

observed. 42% cells arrested/failed cytokinesis and became binucleate (Figure 5.9. and 5.10.). The significant increase in failed mitotic cells upon vacuolin-1 treatment is consistent with lysosome exocytosis during normal cell division. The specificity of vacuolin-1 with regards to lysosome exocytosis has been questioned in studies by Huynh and Andrews. In their work it was determined that even though vacuolin-1 drastically altered lysosome morphology it had no detectable effect on lysosome fusion (C. Huynh & Andrews, 2005). In a more recent study examining autophagy it was suggested that vacuolin-1 inhibited the V-ATPase, thereby neutralizing lysosomal pH and preventing autophagosome-lysosome fusion via Rab5a GTPase activation (J. Yue & Lu, 2014). Lysosomal pH neutralisation was not evident in the results presented in this thesis, since LysoTracker® is present throughout the imaging of vacuolin-1 treated cells (Figure 5.9.). However control experiments were conducted to ensure no obvious morphological effects on endosomes upon vacuolin-1 treatment. No obvious changes in endosomes (Rab5a) could be seen, whereas a severe alteration in lysosome structure (LAMP1) was visible (Figure 5.11. A, B). The data presented in this thesis are therefore consistent with a model of vacuolin-1 activity whereby lysosomes are specifically targeted by this agent with endosomes remaining unaffected. Since the behaviour of vacuolin-1 in different studies has generated conflicting results further analyses are required to determine whether the mitotic failure is due to loss of lysosome fusion with the plasma membrane. Endosomes have been previously suggested to act as membrane donors during mitosis (Boucrot & Kirchhausen, 2007), however, the fact that vacuolin-1 treatment as described in this thesis specifically

affects lysosomes and not endosomes and furthermore that this drug elicits a significant inhibition of normal mitosis suggests an alternative model whereby lysosomes are the primary source of additional membrane during normal mitosis.

One way to define whether lysosome fusion is occurring during cell division is to stain non-permeabilised mitotic cells with an antibody targeting the luminal domain of LAMP1. This approach has been previously performed for plasma membrane repair studies, where the appearance of the luminal portion of LAMP1 at the cell surface is a hallmark of lysosomal fusion with the membrane (A. Reddy et al., 2001). Therefore if luminal LAMP1 staining could be observed on mitotic cells this would be consistent with lysosome exocytosis during cell cycle progression. Another method to study lysosome exocytosis would be to perturb synaptotagmin VII function as this Ca^{2+} sensor has been shown to be present on lysosomes and to regulate lysosomal exocytosis (Martinez et al., 2000) (Rao et al., 2004; A. Reddy et al., 2001). Synaptotagmin VII is a Ca^{2+} sensing protein with a C₂A domain (C. Li et al., 1995) that interacts with membrane phospholipids and SNARE complexes to facilitate membrane fusion (Tucker & Chapman, 2002). Lysosome exocytosis has been demonstrated to be inhibited upon knockdown of synaptotagmin VII (shRNAi targeting) or by blocking the functional C₂A domain of synaptotagmin VII with recombinant peptides (A. Reddy et al., 2001). Further on synaptotagmin II has been studied for its negative effect on lysosome fusion, therefore overexpression of this protein could be employed as well (BARAM 1999). Additionally tetanus neurotoxin-insensitive vesicle-associated membrane protein (TI-VAMP/ VAMP7), a

vesicle SNARE, is part of the lysosome exocytosis machinery via its associated with synaptotagmin VII (Rao et al., 2004). The expression of the Longin domain of TI-VAMP7 has been shown to prevent lysosome fusion and this could also be utilised here (Proux-Gillardeaux, Raposo, Irinopoulou, & Galli, 2007). Employing these methods and observing expressing cells during division would allow robust evidence of lysosomal fusion events requirement during mitosis.

Another hypothesis could be that lysosomes need to be aligned and clustered at either side of the nuclei to act as a checkpoint that once successfully completed signals progression to the next cell cycle stage. Similar to Golgi fragmentation assisting as a cell cycle progression signal, the clustering of lysosomes might be a recognised signal for the cell to move on to the next stage (Colanzi & Corda, 2007). Since CaBP7 knockdown alters the distinctive lysosome localisation the mitotic/cytokinetic checkpoint signal is lost and therefore cells fail to divide. In this theory CaBP7's role would be to correctly localise lysosomes at telophase/cytokinesis. Lysosomes might be required to be at this specific location for perfect segregation to the two daughter cells. This would also be in agreement with the observation that none of the drug treatments except for vacuolin-1 and GPN elicited a mitotic defect. GPN and vacuolin-1 alter lysosome morphology (Figure 5.6. A and Figure 5.11. A), which could interfere and prevent clustering phenomenon during telophase. A simple assay to investigate this theory would be to treat cells with GPN and vacuolin-1, fix and stain for lysosomes and image cells at cytokinesis to evaluate whether lysosome clustering is obstructed.

Pharmacological inhibition of selected lysosome activities is not the only method to investigate a requirement of lysosomes during mitosis. Additional studies could be performed to strengthen and support these results and to broaden the understanding of lysosome function during mitosis. The discovery of lysosome mislocalisation causing a cytokinesis failure has opened a new chapter in the functional biology of lysosomes, which certainly requires much more attention.

5.4. Summary

This chapter aimed to obtain insights into lysosome functions during cell division. By employing lysosomally targeted Ca^{2+} sensors the role of lysosomal Ca^{2+} signalling during cell division was examined. No Ca^{2+} signals in close proximity to lysosomes could be observed during mitosis. Excluding a lysosomal Ca^{2+} signalling role during cell division, other potential lysosome specific functions were explored. By utilizing commercially available compounds specific lysosome functions were perturbed and cells studied during mitotic progression. Bafilomycin and folimycin, compounds known to collapse lysosomal ΔpH and therefore prevent autophagosome- lysosome fusion, did not influence cell division. Similar results were obtained by applying pepstatin A, which is known to inhibit the main lysosomal proteolytic enzyme, Cathepsin D. However vacuolin-1 treatment led to an increased rate of mitotic failure. Vacuolin-1 has been shown to inhibit lysosomal exocytosis with the plasma membrane. These findings suggest that lysosomes might play a membrane donation function during cell division.

Chapter 6

Main Discussion

The CaBPs are a family of EF hand containing Ca^{2+} sensors related to the ubiquitously expressed Ca^{2+} sensing protein CaM (Haeseleer, Imanishi, Sokal, Filipek, & Palczewski, 2002). CaBPs are relatively recently discovered proteins consisting of 9 family members (including CaBP1 and 2 splice variants), further sub divided into CaBPs 1-5 and CaBP7 and 8 (Haeseleer et al., 2000). This sub categorisation is based on the distribution of active and inactive EF hands and the unique 38 amino acid extension found at the C-terminal of CaBP7 and 8 (McCue, Haynes, & Burgoyne, 2010a; McCue, Burgoyne, & Haynes, 2009; McCue, Haynes, & Burgoyne, 2010b). CaBPs 1-5 have been established to regulate various VGCCs, whereas CaBP7 and 8 have been shown to negatively regulate PI4KIII β on the TGN at resting cytosolic Ca^{2+} levels (Mikhaylova et al., 2009). PI4KIII β is a cytosolic kinase which phosphorylates PI to PI4P (A. Balla & Balla, 2006) that can associate with the TGN through interaction with effectors including Arf1 and NCS-1 (Godi et al., 1999; Haynes, Thomas, & Burgoyne, 2005). PI4P is the major precursor for PI (4,5) P_2 but itself is essential in the biogenesis of secretory vesicles that exit from the TGN and transport cargo to the plasma membrane (A. Balla & Balla, 2006; Tan & Brill, 2014). Functional data regarding CaBP7 and 8 is sparse but includes: 1) The aforementioned negative regulation of PI4KIII β and 2) CaBP7 only has been identified in a high-throughput RNAi screen to be important for normal completion of mitosis and cytokinesis (Neumann et al., 2010).

This thesis was designed to further understand CaBP7 functions in cell division. Data presented here demonstrate CaBP7 to associate with lysosomes in addition to the Golgi complex and plasma membrane

(Rajamanoharan et al., 2015). This localisation exhibits cell-cycle dependency and an outstanding question for future studies is how CaBP7 moves from Golgi to lysosomes and back again and what might regulate this novel and dynamic distribution. This novel observation precipitated an investigation of CaBP7 function on lysosomes. Parallel to these studies Sridhar *et al.* published a report characterising a new and biochemically distinct pool of lysosome resident PI4KIII β (Sridhar et al., 2013), which was important for lysosome content sorting. Regulation of this lysosome resident enzyme pool by CaBP7 was therefore tested and results suggested a similar mode of regulation as observed at the TGN. Overexpression of wild type CaBP7 and CaBP7 EF hand mutants (known inhibitors of PI4KIII β activity) led to lysosome clustering in a perinuclear region, similar to the phenotype observed on PI4KIII β knockdown (Sridhar et al., 2013). These results suggest that CaBP7 can be both Golgi and lysosome localised and that it can inhibit PI4KIII β activity in a Ca²⁺ independent fashion on both organelles (Mikhaylova et al., 2009). These results reveal new insights into CaBP7 localisation and extend our knowledge on its cellular functions. The next part of this study focused on the requirement of CaBP7 during mitosis. Down regulation of CaBP7 elicited an accumulation of binucleate cells as previously demonstrated (Neumann et al., 2010), suggesting a role for CaBP7 in telophase or cytokinesis. Consistent with these observations and linking the CaBP7 effect on mitosis to its only known cellular target, PI4KIII β , CaBP7 was able to regulate PI4P levels at all stages of the cell cycle, including mitosis. In agreement with these data, activation of PI4KIII β generated the same cytokinesis failure and binucleate phenotype observed

on CaBP7 depletion. These data led to a more detailed investigation of CaBP7 localisation during the cell cycle. These analyses revealed that CaBP7 along with lysosomes and PI4KIII β displayed a striking re-distribution and clustering to either side of the nuclei during telophase. Interestingly, the distinct lysosome redistribution at telophase was lost upon CaBP7 knockdown. Since the down regulation of CaBP7 caused failure of lysosomes to cluster during cytokinesis, in addition to binucleate cell accumulation, the question arose as to whether cytokinesis failure upon CaBP7 knockdown (KD) was caused by lysosome mislocalisation. In this hypothesis lysosomal clustering is posited to be somehow essential during mitosis and led to the studies detailed in chapter 5 of this thesis, where various lysosomal functions were investigated during cell division. Only prevention of lysosomal fusion had effects on cell division whereas lysosomal Ca²⁺ signalling and proteolytic activity appeared dispensable. Vacuolin-1, a drug previously demonstrated to inhibit lysosomal exocytosis, exhibited a profound effect on cell division however data obtained with this compound should be treated with a degree of caution at present, as there are reports in the literature regarding other potential targets of vacuolin-1 in the cell. Data on vacuolin-1 are therefore consistent with a potential requirement for lysosome exocytosis during cytokinesis however further work needs to be performed, as outlined in chapter 5, before this can be verified. It should be noted that GPN, a second compound that inhibits lysosome fusion, also elicited detectable morphological changes in lysosomes (albeit less severe than those observed with vacuolin-1) and induced significant mitotic failure. The enlarged lysosomes generated upon GPN and vacuolin-1 application, in

addition to inhibiting lysosome fusion events, might also conceivably interfere with normal lysosomal trafficking, particularly to sites flanking the nuclei during telophase. This phenotype would mirror that observed with CaBP7 knockdown and could account for, lysosome mislocalisation at telophase and the subsequent accumulation of binucleate cells. These analyses are essential future work, which would provide more concrete evidence for a fusogenic role of lysosomes during cytokinesis in a spatially restricted zone. Effects on lysosome trafficking (lysosome clustering) were observed upon CaBP7 OE or inactivation of PI4KIII β (Chapter 3, (Sridhar et al., 2013). In contrast, CaBP7 depletion prevented lysosome clustering (Chapter 4). Collectively, these findings hint at a mechanism whereby CaBP7 controls lysosome trafficking through PI4KIII β regulation.

Membrane bound organelles move along cytoplasmic microtubules by the action of mechano-chemical motor proteins. Microtubule-based lysosome movement was first discovered in 1984 (Herman & Albertini, 1984; MATTEONI & KREIS, 1987) and has since been studied in greater detail. Minus end directed, also known as centripetal (inward), movement towards the microtubule organization centre (MTOC), involves the small GTPase Rab7 and its effector Rab7 interacting lysosomal protein (RILIP). RILIP recruits dynein- dynactin minus end microtubule motors permitting trafficking towards the perinuclear region (Cantalupo, Alifano, Roberti, Bruni, & Bucci, 2001; Jordens et al., 2001). Conversely, plus end directed, or centrifugal (outward), movement is facilitated by the GTPase Arl8b and its effector protein SifA and kinesin interacting protein (SKIP), which link lysosomes to kinesin-1, the plus end directed microtubule motor protein (Bagshaw,

Callahan, & Mahuran, 2006; Hofmann & Munro, 2006; Rosa-Ferreira & Munro, 2011). Centripetal (Rab7 regulated) and centrifugal (Arl8b regulated) directional movement is summarised schematically in diagram 6.1.

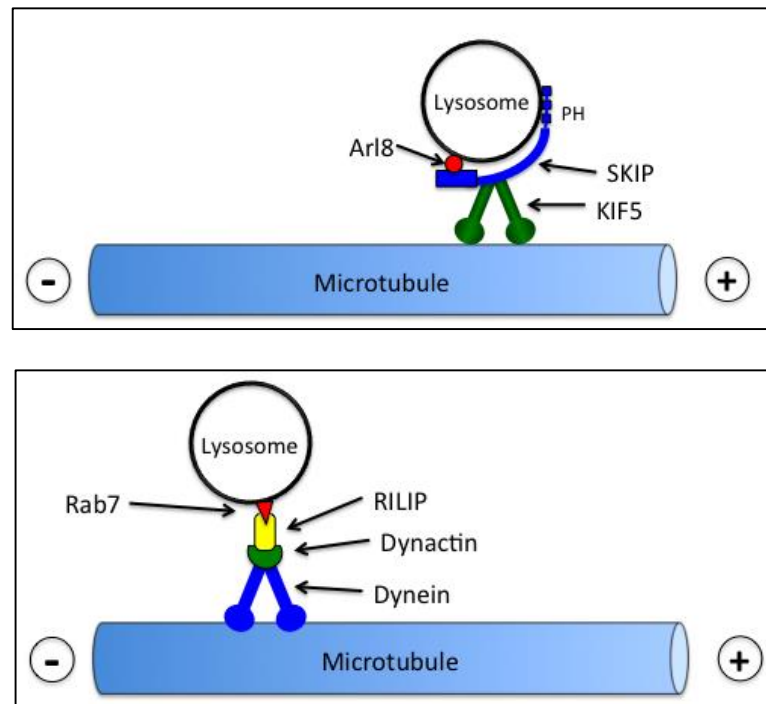


Diagram 6.1. Lysosome trafficking along microtubules. Top schematic illustrates the interaction of KIF5, SKIP and Arl8b facilitating centrifugal mobility of lysosomes (+ end directed movement). Bottom schematic shows the molecular machinery comprising dynein-dynactin, Rab7 and RILIP, which interact to mediate centripetal movement of lysosomes toward the MTOC (- end directed movement).

SKIP is a soluble cytosolic protein containing RUN and Pleckstrin homology (PH) domains (Rosa-Ferreira & Munro, 2011). Interestingly the phosphoinositide binding preference of SKIP's PH domain remains unknown as does its role in lysosome trafficking. PH domain/phosphoinositide interactions typically result in protein recruitment and cellular targeting to specific membrane domains Residues 1-300 at the N-terminal of SKIP

(comprising the RUN domain) are sufficient to interact with Arl8b, but peripheral lysosome distribution is lost. The truncated SKIP lacking this domain (SKIP 301- 1019 including the PH domain) is completely cytoplasmic and unable to associate with lysosomes (Rosa-Ferreira & Munro, 2011). These findings imply the importance of Arl8b association via the N-terminal of SKIP (1-300) but also the necessity of the remaining protein with the PH domain for stable membrane association with lysosomes for peripheral anchoring. The requirement of SKIP for Arl8b regulated lysosome trafficking was further confirmed by performing silencing and OE of SKIP in combination with Arl8b.

Cellular depletion of SKIP led to accumulation of lysosomes at the perinuclear region suggesting disruption of coupling to kinesin-1 driven + end directed movement which could not be rescued by Arl8b overexpression.

Conversely SKIP OE could rescue Arl8b depletion triggered lysosome clustering indicating a critical function for SKIP in regulating kinesin dependent lysosome trafficking (Rosa-Ferreira & Munro, 2011). The results obtained from work presented in chapters 3 and 4 combined with the published SKIP studies leads to an attractive hypothesis for CaBP7's possible role in regulating lysosome mobility during mitosis. CaBP7 regulates PI4KIII β on lysosomes, hence controlling lysosomal PI4P levels, which in turn could determine SKIP –lysosome association via its PH domain. The assumption here is that SKIP's PH domain binds specifically to PI4P lipid. It should be noted that examples of PI4P specific PH domains have been identified in recent years (Hammond, Machner, & Balla, 2014). In this model, low PI4P levels, induced by CaBP7 inhibition of PI4KIII β , would decrease

SKIP association with lysosomes, therefore lysosomes would lose their anchor in the cell periphery and would become available for migration to the perinuclear region (clustering). Increased PI4P levels would correspondingly mediate association of SKIP with lysosomes, thus mediating outward movement (peripheral vesicular distribution). Consistent with this model, results from chapter 3 demonstrated that inhibition of PI4KIII β upon CaBP7 OE (reduced PI4P levels), eliciting lysosome clustering at the microtubule organisation centre (MTOC). Conversely evidence from chapter 4 examining CaBP7 depletion (increased PI4P levels) demonstrated a dispersed lysosome distribution instead of clustering to either side of the nuclei. The theory of PI4P levels regulating the SKIP-lysosome association certainly is in agreement with the findings in this thesis (Diagram 6.2.). Constitutive activation of PI4KIII β by CaBP7 knockdown and OE of PI4KIII β activators induced cytokinesis failure. However the loss of lysosome clustering at telophase was only investigated in CaBP7 silenced cells, and not in cells overexpressing PI4KIII β activators. If PI4KIII β activator overexpression were also to impair lysosome clustering, then this would indicate that PI4KIII β activity does indeed regulate lysosome mobility. To establish this, PI4KIII β activator transfected cells could be fixed and stained with antibodies directed toward lysosomal markers and cells at telophase analysed for the distribution of lysosomes.

This model of lysosome trafficking may represent an over-simplification however as it appears that there are additional protein complexes that can influence lysosome targeting.

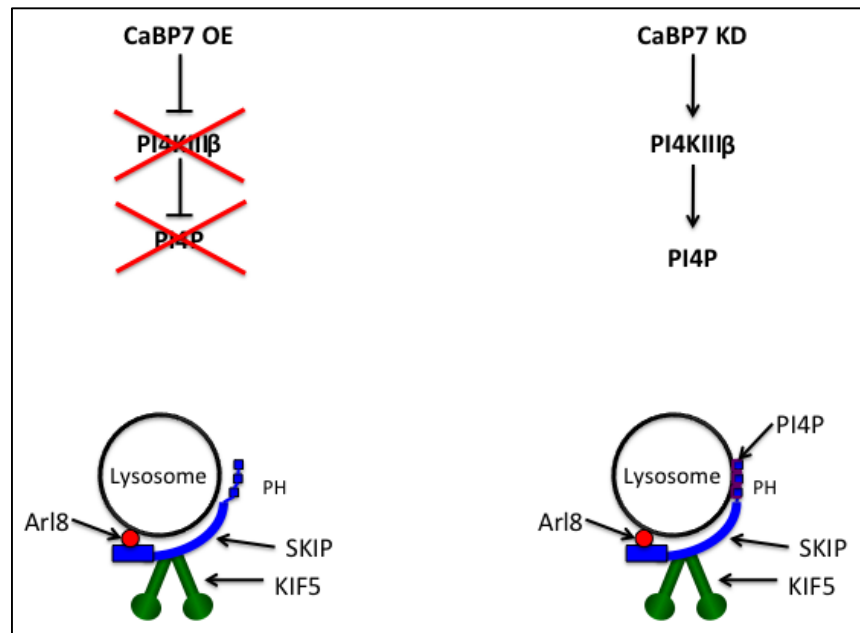


Diagram 6.2. Theoretical model of CaBP7 influencing lysosome motility.

On the left, CaBP7 overexpression leads to PI4KIII β inhibition, hence PI4P levels are reduced. Assuming that the SKIP PH domain binds to lysosomal PI4P, this could mean that SKIP cannot fully associate with lysosomes, resulting in lysosomes clustering at the MTOC as seen in chapter 3. On the right, upon CaBP7 knockdown PI4KIII β will be constitutively active leading to increased lysosomal PI4P. Again, assuming PI4P levels mediate SKIP PH and lysosome association, this will result in a tight and robust interaction of SKIP with lysosomes and result in peripheral movement of lysosomes as detailed in chapter 4.

In a recent paper by Pu *et al.*, the role of Bloc-one related complex (BORC), was investigated and shown to be involved in lysosome positioning and movement (Pu *et al.*, 2015). Similar to the phenotype observed when SKIP is depleted from cells, silencing of BORC subunits elicited lysosome clustering and a loss of Arl8b association with lysosomes indicating a disruption in kinesin dependent transport to the cell periphery. Identically to studies of SKIP and Arl8b, it was also shown that Arl8b overexpression could not

rescue the lysosome clustering phenotype observed on depletion of certain BORC subunits, in particular Myrlysin (Pu et al., 2015). Consistent with these studies (Pu et al., 2015; Rosa-Ferreira & Munro, 2011), observations from preliminary experiments exploring Arl8b in CaBP7 overexpressing cells highlight that lysosome clustering at the juxtanuclear region was associated with a cytosolic, not vesicular, localisation of Arl8b compared to control cells. This experimental data strengthens the novel idea of CaBP7's involvement in the Arl8b-SKIP/BORC-Kinesin1 dependent pathway of lysosome trafficking. To support the hypothetical model presented above it is imperative to identify the lipid binding specificity of the SKIP PH domain. The majority of identified PH domains bind phosphoinositides with low affinity and with limited specificity. PH domain binding with high affinity and specificity has only been shown for PI (4,5) P₂, PI (3,4) P₂ and PI (3,4,5,) P₃. However lipid overlay experiments utilizing lipid strip blots, with spotted phosphoinositides could be employed to reveal new insights into the binding specificity of the SKIP-PH domain (Lemmon, 2007). Further experiments examining the effect on Arl8b-SKIP- kinesin1 pathway, with CaBP7 and inactive PI4KIII β (PI4KIII β ^{A656D}) would also be valuable. Considering that loss of lysosome clustering is associated with an increased frequency of binucleate cells in CaBP7 depleted cells, rescue experiments to counteract the lysosomal centrifugal movement could be performed. CaBP7 shRNAi expression in combination with Rab7 OE could potentially rescue the binucleate phenotype by correcting the lysosome localisation defect. Dual-knockdown of both CaBP7 and SKIP could also be analysed for cytokinesis failure, to appreciate whether the down regulation of SKIP can also salvage the loss of lysosome

clustering at telophase. Additionally other combinations of KD plus OE could be tested to rescue the lysosome clustering phenotype upon CaBP7 OE observed in chapter 3. CaBP7/SKIP co-expression or CaBP7 OE and Rab7 KD could be analysed for lysosome distribution. In conclusion, extended studies should be performed to clarify the precise role of CaBP7 in regulating lysosome movement.

If CaBP7 regulates PI4P levels on lysosomes and it is this that determines SKIP association, more sophisticated tools and techniques could be applied to investigate this. KD and OE studies last 24-72 hours, whereby cells might possibly adapt to compensate for the absence or presence of a particular protein. This is perhaps an important issue when investigating the cellular functions of an enzyme like PI4KIII β as there are three other PI4K isoforms expressed by mammalian cells. Acute pharmacological inhibition is an attractive approach but relies on the availability of selective inhibitors which have only recently emerged for PI4KIII β and have not been extensively tested in cell systems (Mejdrová et al., 2015). A more sophisticated and powerful method to increase or decrease phosphoinositides at specific cellular compartments is available and could be utilised in future work. Balla *et al.* exploited the principal of the 12kDa FK506 binding protein (FKBP) and FKBP12-rapamycin binding (FRB) domain. FRB-fused to specific organelle resident motifs can recruit FKBP-conjugated specific phosphoinositide kinases or phosphatases upon rapamycin induction, thus permitting precise modulation of phosphoinositide levels at spatially discrete locations. This system also has the advantage that changes to phosphoinositide levels can be induced relatively rapidly thereby circumventing issues (as discussed

above) that may arise from cellular adaptation. FRB- Rab7 (for lysosome targeting), FKBP- Sac1 (dephosphorylates PI4P to PI, hence depletes PI4P) and FKBP- INPP5 (converts PI (4,5) P₂ to PI4P, thus increases levels of PI4P) plus a PI4P biosensor probe GFP-P4M are all readily available (Hammond et al., 2014). All constructs are fused to different fluorophores ranging from ultraviolet to infrared, enabling imaging of all constructs simultaneously. Experiments employing these constructs could provide direct evidence whether the fine-tuning of PI4P on lysosomes defines lysosome trafficking and disrupts cell division. If lysosome positioning to either side of the nuclei is an essential step for cytokinesis completion, further studies, including use of the rapamycin inducible system described above, are required (in an extension to chapter 5) to understand whether lysosomes exert a unique functional role at this stage. Another possibility would be that the clustering of lysosomes at this stage represents a required signal for cell cycle progression and exact lysosome segregation into the new daughter cells.

Interestingly possible involvement of CaBP7 in lysosome trafficking presents to some extent comparisons with regulation of mitochondrial movement. Mitochondrial Rho (Miro) GTPases, Miro-1 and Miro-2 are EF-hand containing tail-anchored membrane proteins localised on the outer mitochondrial membrane. These proteins have been shown to exist in a complex with Milton (TRAK1/2), kinesin-1 and dynein, which is directly responsible for mitochondrial trafficking (Fransson, Ruusala, & Aspenstom, 2006). Even though the involvement of CaBP7 on lysosomes trafficking is certainly not the same, as there is no evidence of direct association of

CaBP7 to either kinesin1 nor dynein, but the results insinuate an indirect link of lysosome resident CaBP7 to regulate PI4KIII β preceding lysosome trafficking. Considering CaBP7 is a tail-anchored and EF hand containing protein possibly interacting with the GTPase Arl8b involved in lysosome localisation certainly reveals similarities to Miro-1 and 2.

CaBP7, as an EF hand containing protein, will likely possess Ca²⁺ dependent functions. So far the identified interaction with PI4KIII β on the TGN and lysosomes seems Ca²⁺ independent, since even EF hand mutated CaBP7 can regulate PI4KIII β activity. Extended protein- protein interactions studies in the presence/ absence of Ca²⁺ as well as with EF hand mutants and wild type protein should be performed for CaBP7 in order to identify novel interaction partners and especially those that are Ca²⁺ dependent. Various methods such as co-immunoprecipitation, pull-down assays or cross-linking methods can be applied (Miernyk & Thelen, 2008) followed by mass spectrometry of candidate prey proteins.

Conclusion

Data presented in this thesis has aimed to provide a more detailed understanding of the cellular roles of CaBP7. Over the course of these investigations a number of novel observations have been made which have led to entirely new insights into CaBP7 function. Firstly, in addition to exhibiting a TGN localisation CaBP7 has been shown to also reside on lysosomes and this localisation appears cell-cycle dependent. The second novel finding from this work is that at least one role of lysosomal CaBP7 is to

regulate the lysosomal specific pool of PI4KIII β . The requirement for CaBP7 during mammalian cell division was established as previously reported. These investigations led to a third novel finding, namely that CaBP7 along with lysosomes and PI4KIII β demonstrate a striking re- distribution to either side of the nuclei at telophase. Work in this thesis has also demonstrated that CaBP7 can regulate PI4KIII β activity, thus PI4P levels, throughout all stages of the cell cycle. In related studies a fourth major novel finding involved the demonstration that CaBP7 knockdown not only caused an accumulation of binucleate cells but also induced loss of lysosome clustering at telophase. These data led to a working hypothesis that unique lysosome positioning during cytokinesis was in some way essential for normal completion of mitotic cell division. Pharmacological dissection of lysosome function led to a fifth novel finding that suggests a possible role of lysosome exocytosis during mitosis and which provides a focus for future studies.

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Appendix

(CD attached to the inside of the back cover)

Videos accompany the following still images

Figure 4.7.

Co-localisation of exogenous CaBP7 and LAMP1 during mitosis p. 141

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CaBP7 depletion and loss of lysosome clustering p. 156

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Inhibition of V-ATPase activity during cell division (Bafilomycin) p. 195

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